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(54) Title: MODIFICATION OF LIGNIN SYNTHESIS IN PLANTS

(57) Abstract

The biosynthesis of lignin in plants is regulated by insertion into the plant genome by altering the plant's ability to synthesize the enzyme O-methyl-transferase, an enzyme involved in the lignin biosynthetic pathway. Production of O-methyl-transferase may be enhanced by insertion into the plant genome by transformation of one or more additional copies of the O-methyl-transferase gene or production may be inhibited by insertion of a gene encoding anti-sense mRNA directed against the mRNA encoded by the endogenous O-methyl-transferase gene.

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MODIFICATION OF LIGNIN SYNTHESIS IN PLANTS

This invention relates to the improvement of plants by the modification of lignin biosynthesis, particularly, but not exclusively, the improvement of digestibility of fodder crops.

Grassland farmers, and farmers of other fodder crops, face a difficult decision each year about when to cut their crops for conservation. All grass varieties of agricultural importance suffer from the disadvantage that during the normal increase in dry matter yield with growth, the digestibility decreases. The farmer, therefore, has, to compromise between a lower yield of highly digestible material and a higher yield of less digestible material. Another limitation is that harvesting at optimum maturity may be prevented by unfavourable weather. If the decline in digestibility could be controlled or delayed, higher yields of highly digestible material could be obtained and the prevailing weather conditions would not play such a major role in determining the quality of the harvested crop.

Digestibility of fodder crops is determined, among other factors, by the amount and quality of lignin deposition which has taken place during growth of the plants and the degree of secondary modification of lignin deposited. Beside cellulose and other poly- saccharides, lignins are an

essential component of cell wall in tissues like the sclerenchyma and the xylem of vascular plants. They play an important role in the conducting function of the xylem by reducing the permeability of the cell wall to water. They are also responsible for the rigidity of the cell wall, and, in woody tissues, they act as a bonding agent between cells, imparting to the plant a resistance towards impact, compression and bending. Finally, they are involved in mechanisms of resistance to pathogens by impeding the penetration or the propagation of the pathogenic agent.

Lignins are not only important in the productivity and performance of field crops but are also of great importance in trees for paper making. Considerable energy and chemical input is required to loosen, dissolve and remove lignin from the cellulose fibre which is required for paper making. In addition to these instances in which lignins present a constraint on the use of crop plants, lignins are also used as feedstocks for the preparation of speciality chemicals such as phenolics which can be used as precursors in chemical synthesis. Thus lignins and their biological and chemical modification are important.

It is one of the objects of the present invention to provide a biotechnological procedure for the modification both lignin content and lignin composition in plants.

Lignins are the product of a dehydrogenative polymerisation of three primary precursors: the trans-coniferyl, trans-sinapyl and trans-p-coumaryl alcohols. The monomers can occur in lignins in different proportions and with different types of

links both with each other and with the surrounding cell wall polysaccharides,thus producing a wide variety of polymers. These polymers, or "lignin cores" are always associated covalently with hemicelluloses. Most lignins also contain varying amounts of aromatic carboxylic acids in ester-like combinations. Such differences in the structure of lignins are usually found in plant species. However, differences in the composition of lignins, and even in the binding to the primary and secondary cell walls, can also occur in the same plant, between different tissues of different ages. The biosynthesis of lignin monomers (monolignols) is a part of the phenylpropanoid biosynthesis pathway, which is also responsible for the production of a wide range of compounds including flavonoid pigments, isoflavonoids, coumarin phytoalexins and cell division promoting dehydrodiconiferyl glucosides.

Phenylalanine is deaminated to produce cinnamic acid. This acid is then transformed by hydroxylation and methylation reactions, thus producing different acids substituted on the aromatic ring. The enzyme catalysing the methylation steps is O-methyl transferase (OMT). O-methyltransferases (S-adenosyl-L-methionine: O-methyltransferases; EC 2.1.1.6) thus play an important role in the biosynthesis of monolignols. By the O-methylation of caffeic acid and 5-hydroxyferulic acid, OMTs introduce one and two methoxy groups in the lignin monomers, respectively. The resulting two phenolics, ferulic acid and sinapic acid, respectively, are the precursors of coniferyl alcohol and sinapyl alcohol

which are together with coumaryl alcohol substrates for peroxidases (Lewis and Yamamoto, 1990).

The previous methylation reactions are also used in the synthesis of several other phenolic compounds. However, in those cells which are dedicated to the production of lignins such as vascular xylem cells of plants, the OMT plays a crucial role in the production of the phenolic precursors incorporated into the lignin polymer. The cinnamyl alcohols, synthesised in the cytoplasm, are then transported to the cell wall where they are polymerised by peroxidase in the presence of hydrogen peroxide.

When the surface growth of the cell ceases, it is followed by a phase of wall thickening (secondary wall formation). Lignification takes place during this phase. It starts in the cell corners and extends along the middle lamella, through the primary wall and, finally, to the secondary wall. External factors can induce qualitative and quantitative modifications in lignification. The synthesis of new types of lignins, sometimes in tissues which are not normally lignified, may be induced by infection with pathogenic microorganisms. Lignification is stimulated by light, as well as by low calcium levels, by boron, by mechanical stress and by infection.

As a first step in unravelling of lignin biosynthesis at the molecular level, we have undertaken the biochemical characteristics and cloning of O-methyltransferases (OMTs). Previously three different OMTs (OMT I, OMT II, and OMT III) have been purified from tobacco. OMT I used mainly

caffeic acid and 5-hydroxyferulic acid as a substrate and is the OMT actively present in healthy plants. OMT II and OMT III have a broader substrate specificity and also use catechol as substrate. Upon infection with TMV, an increase in activity of all three OMTs was shown. Based on this observation, it has been postulated that the OMT I is specifically involved in lignification, whereas OMT II and OMT III have a function in generating a lignin barrier upon infection. The importance of methylation in monolignol biosynthesis is well illustrated in brown-rib corn mutants. These plants exhibit a reduced lignin content and accumulate 5-hydroxyferulic acid) due to a low O-methyltransferase activity. Thus OMTs could be potential targets for modulation of lignification through the use of recombinant DNA technology.

Thus, plants with a reduced amount of lignin would be more efficiently used as a forage for cattle. The yield of milk and meat would be therefore increased. Furthermore, lignin may have a negative effect on plant growth. Thus, a reduction of the lignification in crops such as wheat, oilseed rape, sugar beet or maize might presumably increase the grain yield. Trees with reduced lignin contents or altered lignin structure will lead to a reduction in the cost of the paper as less lignin will have to be removed during the pulping process. On the other hand, novel papers may be produced due to the purity of cellulose fibre which could otherwise not be produced.

Reduction of lignification can be achieved by the application of chemical inhibitors to plants.

However, a more effective method controlling lignin deposition and structure is the inhibition of expression of the CAD gene using antisense RNA. Antisense RNA technology is an appropriate molecular biology approach to the inhibition of lignification. An antisense RNA is an RNA produced by the transcription of the non-coding DNA strand (nonsense). Thus, antisense RNA has the same sequence as the coding DNA strand and is complementary to the mRNA product of a specific gene.

As is well known, a cell manufactures protein by transcribing the DNA of the gene for that protein to produce RNA, which is then processed (e.g. by the removal of introns) into messenger RNA and finally translated by ribosomes into protein. This process may be inhibited by the presence in the cell of "antisense RNA". Therefore, as used herein, the term "antisense RNA" means an RNA sequence which is complementary to a sequence of bases in a mRNA: complementary in the sense that each base (or a majority of bases) in the antisense sequence (read in the 3′ to 5′ sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5′ to 3′ sense. It is believed that this inhibition takes place by formation of a complex between the two complementary strands of RNA, preventing the formation of protein. How this works is uncertain: the complex may interfere with further transcription, processing, transport or translation, or lead to degradation of the mRNA, or have more than one of these effects. Such antisense RNA may be produced in the cell by transformation with an

appropriate DNA construct arranged to transcribe backwards part of the coding strand (as opposed to the template strand) of the relevant gene (or of a DNA sequence showing substantial homology therewith).

The use of this technology to down-regulate the expression of specific plant genes has been described, for example in European Patent Publication No 271988 to ICI. Reduction of gene expression has led to a change in the phenotype of the plant: either at the level of gross visible phenotypic difference e.g. lack of lycopene synthesis in the fruit of tomato leading to the production of yellow rather than red fruit or at a more subtle biochemical level e.g. change in the amount of polygalacturonase and reduction in depolymerisation of pectins during tomato fruit ripening (Smith et al, Nature, 334, 724-726, 1988; Smith et al, Plant Mol Biol 14, 369-380, 1990). Thus antisense RNA has been proven to be useful in achieving down-regulation of gene expression in plants.

An object of the present invention is to provide plants having an altered ability to synthesise lignin.

According to the present invention there is provided the DNA insert contained in the clones pPLC4 and pTOMTI and variants thereof such as are permitted by the degeneracy of the genetic code or the functional equivalents thereof.

In addition, the present invention provides a recombinant DNA construct containing the said DNA under control of a transcriptional control sequence operative in plants, so that the construct can

generate mRNA in plant cells.

For the down-regulation of lignin synthesis the aforesaid DNA is in antisense orientation.

For the amplification of lignin biosynthesis the aforesaid DNA is in sense orientation thus to provide one or more additional copies of the said DNA in the plant genome.

Thus, in a further aspect, the present invention provides DNA constructs comprising a transcriptional initiation region operative in plants positioned for transcription of a DNA sequence encoding RNA complementary to a substantial run of bases showing substantial homology to an mRNA encoding the protein produced by the gene in pPLC4 pTOMT1.

The invention further provides plant cells, and plants derived therefrom having stably incorporated in their genomes the aforesaid DNA in sense or antisense orientation, and fruit and seeds of such plants. The present invention is principally concerned with the suppression of lignin formation and, that being so, the inserted gene will be in antisense orientation, but there are instances where over-production of lignin may have an advantageous effect, for example to improve plant stalk strength and resistance to diseases, and the present invention provides means for achieving amplification of the lignin biosynthetic ability of plants.

Thus the invention relates generally to the regulation of the plant's lignin biosynthetic pathway, in which OMT plays a dominant role, in order that the production of OMT, and hence the production and composition of lignin is altered by

insertion of the OMT gene, or a portion thereof (usually of 50 or more bases), in antisense orientation so that the amount of OMT for catalysing lignin synthesis is reduced.

The constructs of the invention may be inserted into plants to regulate the production of the CAD enzyme. Depending on the nature of the construct, the production of the protein may be increased, or reduced, either throughout or at particular stages in the life of the plant. It is also possible to target the expression of the gene to a specific cell types of the plant, such as the epidermis, the xylem, the roots etc.

The plants to which the present invention can be applied include commercially important food and forage plants, such as alfalfa, maize, oil seed rape, forage grasses and sunflower, and but also tree crops such as eucalyptus, pine species and poplar.

DNA constructs according to the invention preferably comprise a sequence of at least 50 bases which is homologous to the DNA of the insert in pPLC4 or pOMT1.A and pOMT1.B There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences between 100 and 1000 bases in length. The preparation of such constructs is described in more detail below.

The preferred source of antisense RNA for use in the present invention is DNA derived from the clone pPLC4 or pOMT1.A and pOMT1.B The required DNA encoding antisense RNA can be obtained in several ways: by cutting an appropriate sequence of

DNA from pPLC4 or pOMT1.A or pOMT1.B (or any other source of the OMT gene); by synthesising a DNA fragment using synthetic oligonucleotides which are annealed and then ligated together in such a way as to give suitable restriction sites at each end; by using synthetic oligonucleotides in a polymerase chain reaction (PCR) to generate the required fragment with suitable restriction sites at each end. The DNA is then cloned into a vector containing upstream promoter and downstream terminator sequences, the cloning being carried out so that the DNA sequence is inverted with respect to its orientation to the promoter in the strand from which it was cut. In the new vector, the strand that was formerly the template strand becomes the coding strand, and vice versa. The new vector will thus encode RNA in a base sequence which is complementary to the sequence of pPLC4 and pTOMT1 mRNAs. Thus the two RNA strands are complementary not only in their base sequence but also in their orientations (5' to 3').

As source of the DNA base sequence for transcription, it is convenient to use a cDNA clone such as pPLC4. The base sequence of pPLC4 is shown in Figure 1 and the base sequences of pOMT1.A and pOMT1.B is shown in Figure 2.

The clone pPLC4 has been deposited at the National Collections of Industrial and Marine Bacteria, PO Box 31, of 23 St Machar Drive, Aberdeen AB2 1RY, Scotland, as a plasmid in E.coli, strain 'sure', under the reference NCIB 40436 on August 22, 1991.

The clone pTOMT1.A has been deposited at the National Collections of Industrial and Marine

Bacteria, PO Box 31, of 23 St Machar Drive, Aberdeen AB2 1RY, Scotland, as a plasmid in E.coli, strain DH5α, under the reference NCIB 40439 on September 4, 1991.

The clone pTOMT1.B has been deposited at the National Collections of Industrial and Marine Bacteria, PO Box 31, of 23 St Machar Drive, Aberdeen AB2 1RY, Scotland, as a plasmid in E.coli, strain DH5α, under the reference NCIB 40440 on September 4, 1991.

A source of DNA for the base sequence for transcription is the promoter of the OMT gene itself or other genes involved in lignification such as the promoter of the phenyl alanine ammonia lyase gene or its modified version which permits expression in xylem tissue, or the s-Adenosyl methionine synthase gene or the promoter of the extensin gene. Such a gene may differ from the cDNA of pPLC4 and pOMT1A or pOMT1.B in that introns may be present. The introns are not transcribed into mRNA (or, if so transcribed, are subsequently cut out). When using such a gene as the source of the base sequence for transcription it is possible to use either intron or exon regions.

A further way of obtaining a suitable DNA base sequence for transcription is to synthesise it ab initio from the appropriate bases, for example using Figure 1 and Figure 2 as a guide. Recombinant DNA and vectors according to the present invention may be made as follows. A suitable vector containing the desired base sequence for transcription (for example pPLC4 and pOMT1.A and pOMT1.B) is treated with restriction enzymes to out cut the sequence. The DNA strand so obtained is

cloned (in reverse orientation) into a second vector containing the desired promoter sequence (for example cauliflower mosaic virus 35S RNA promoter or the bean PAL promoter, Bevan et al, EMBO J.8, 1899-1906 1988) and the desired terminator sequence (for example the 3' of the Agrobacterium tumefaciens nopaline synthase gene.

In this invention we may use both constitutive promoters (such as cauliflower mosaic virus 35S RNA) and inducible or developmentally regulated promoters (such as the PAL gene promoter) as circumstances require. Use of a constitutive promoter will tend to affect functions in all parts of the plant: while by using a tissue specific promoter, functions may be controlled more selectively. The use of a tissue-specific promoter, has the advantage that the antisense or sense RNA is only produced in the tissue in which its action is required.

Vectors according to the invention may be used to transform plants as desired, to make plants according to the invention. Dicotyledonous plants, such as alfalfa, oil seed rape etc, may be transformed by Agrobacterium Ti plasmid technology, for example as described by Bevan (1984) Nucleic Acid Research, 12, 8711-8721. Such transformed plants may be replicated sexually, or by cell or tissue culture.

Poplar and aspen transformation using Agrobacterium tumefaciens can be performed as described by De Block [Plant Physiol. (1990) 93:1110-1116]. Stem internode pieces are used as a tissue source for incubation with an Agrobacterium tumefaciens strain (C58CRif^{R}(pMP90.) harbouring

chimeric marker genes (bar/neo) on its non-oncogenic T-DNA. For the aspen clone (Populus alba x P. tremula; clone 357, Afocel) and the poplar clone (Populus trichocarpa x P. deltoides; clone 064, Afocel), transgenic shoots were obtained 3 months and 6 months after incubation, respectively.

The degree of production of RNA in the plant cells can be controlled by suitable choice of promoter sequences, or by selecting the number of copies, or the site of integration, of the DNA sequences according to the invention that are introduced into the plant genome. In this way it may be possible to modify lignification to a greater or lesser extent.

The constructs of our invention may be used to transform cells of both monocotyledonous and dicotyledonous plants in various ways known to the art. In many cases such plant cells (particularly when they are cells of dicotyledonous plants) may be cultured to regenerate whole plants which subsequently reproduce to give successive generations of genetically modified plants. Examples of genetically modified plants according to the present invention include, alfalfa, oil seed rape, sunflower, sorghum, maize, festuca, and trees such as eucalyptus, poplar, and pine.

In the present invention, we use antisense RNA in order to determine the phenotype of transgenic plants which show modified, that is increased or reduced, expression of pPLC4 or pTOMT1 by the use of antisense and sense expression vectors.

The invention will now be described further with reference to the accompanying drawings, in which:

Figure 1 shows the complete nucleotide sequence and deduced amino acid sequence from pPLC4.

Figure 2 shows the combined nucleotide sequence and deduced amino acid sequence of pOMT1.A and pOMT1.B.

Figure 3 shows the nucleotide sequence of a complete cDNA clone of OMTI from a stem tobacco library.

Figure 4 is the nucleotide sequence of a OMT III cDNA isolated from a λZapII library of tobacco leaf.

Figure 5 shows the construction of antisense and sense vectors using the 5′ end 500 bp and the 3′ end 900 bp BamH1 fragments from pPLC4.

Figure 6 shows the construction of an antisense vector using a 1.4 kb PCR fragment containing the complete pPLC4 clone.

The invention will now be described by way of illustration in the following Examples.

EXAMPLE 1

Purification of poplar OMTs

A. Isolation procedure

Table 1 shows the OMT activity using caffeic acid as a substrate in young leaves and xylem tissue from poplar trees (Populus trichocarpa x P. deltoides).

TABLE 1

OMT Activity in young leaves and xylem tissue from poplar trees

Tissue	Activity/g tissue n kat/g	Specific Activity n kat/g protein
Leaves	0.015	0.002
Xylem	0.6	0.2

There is approximately 50-fold more caffeic acid O- methyltransferase activity in xylem as compared to leaves. Subsequently, OMT activity was purified both from leaves and xylem tissue. The procedure for the purification of the OMTs was established by Dumas et al., (1988). The purification of OMT activity from a total protein extract of poplar leaves via ammonium sulphate precipitation, desalting on Sephadex G25, Q-Sepharose chromatography, and adenosine agarose affinity chromatography, and finally, MonoQ chromatography resulted in one 38-kDa protein. About 5 μg of OMT was obtained from 100 g of leaves. Using xylem as source, several purification steps were omitted. Crude protein extract from 5 g of xylem was applied immediately on an agarose adenosine column leading to the purification of about 50 μg of the 38- kDa OMT with a yield of 50% . At this stage a minor contaminating band of 37-kDa is still visible.

B. Immunological characterisation of poplar OMTs.

Antibodies raised against OMT I and OMT II from tobacco were used to test for cross-reactions

with the poplar OMTs. Antibodies raised against OMT II cross-react with the proteins (Mr 38 kDa and 70 kDa) on protein gel blots of proteins purified from leaves by three purification steps (G25, Q-Sepharose, and adenosine agarose affinity chromatography). Antibodies raised against OMT I show weak cross- reaction with a 37-kDa protein. A fourth purification step (chromatography on MonoQ column) resulted in one 38-kDa protein that cross-reacts solely with antibodies directed against OMT II on protein gel blots.

C. Inhibition in vitro of the poplar OMT caffeic acid O-methyltranferase activity by antibodies against OMT II and OMT I from tobacco.

Although the poplar OMT and the tobacco OMT II have a different substrate specificity, the protein gel blots clearly indicate that both enzymes must have similar epitopes recognised by the rabbit antiserum against tobacco OMT II.

Five ng of purified poplar OMTI was mixed with corresponding amounts of antibodies directed against tobacco OMTI and OMTII. After incubation at 37°C, OMT activity towards caffeic acid was measured. The results are given in Table 2 which shows that the caffeic acid OMT activity of the purified poplar OMT can be inhibited by adding antibodies directed against OMT II from tobacco prior to the OMT activity assay (see Materials and Methods). Two and four μl of undiluted rabbit antiserum against tobacco OMT II mixed with purified poplar OMT resulted in a 87% and 92% inhibition, respectively, of the OMT activity. However, 2 μl of undiluted anti-OMT I rabbit antiserum mixed with purified poplar OMT led to a

29% inhibition of the OMT activity. No inhibition of the OMT activity was observed by mixing 2 µl of pre-immune serum with the purified poplar OMT prior to the OMT activity assay.

TABLE 2

Inhibition in vitro of the poplar OMT activity by antibodies directed against OMTI and OMTII from tobacco

Antibody	OMT activity toward Caffeic acid (%)	Inhibition (%)
-	100	0
2µl anti-OMTI	71	29
2µl anti-OMTII	13	87
4µl anti-OMTII	8	92
2µl pre-immune serum	100	0

D. Specific activity of the purified poplar OMT towards different phenolic compounds.

The specific activity of the purified OMT towards three different O-diphenolic substrates was measured. Using catechol, caffeic acid, and hydroxyferulic acid as substrate, we found an OMT activity of 0, 30, and 15 nkat/mg protein, respectively.

Previously, it has been shown that OMT 1 from tobacco uses mainly caffeic acid and hydroxyferulic acid as a substrate (Collendavelloo et al., 1981). Therefore, the purified poplar OMT has an OMT I-like enzymatic activity.

EXAMPLE 2

Microsequencing of the 38-kDa OMT.

The 38-kDa protein isolated from poplar xylem tissue was digested with trypsin and the peptides were separated on reverse-phase HPLC. Four peptides were sequenced:

peptide 45 (R/KDLPHVIEDAPSYGVEHVGGDMF)

peptide 49 (LVDVGGGTGAVV)

peptide 51 (GINFDLPHVIEDAP)

peptide 52 (VILVE?ILPVAPD).

Note that peptide 45 is a mixture of two peptides with arginine and lysine, respectively, as first amino acid. Trypsin cleaves proteins after a lysine or arginine residue, except when this is followed by proline, glutamic acid, or aspartic acid. This implies that the first amino acid preceding the sequence of peptide 45 has to be either an arginine or a lysine. The sequences of peptide 45 and 51 are overlapping.

EXAMPLE 3

Design of oligos and cloning of OMT sequences.

Two amino acid sequences of peptide 49 (VDVGGGTGA) and peptide 51 (PHVIEDAP) were chosen to design degenerated oligonucleotides which were subsequently used as primers for a PCR with DNA prepared from a leaf cDNA library of poplar. Since the relative position of the peptide in the protein are unknown, both sense and antisense oligonucleotides were designed. Following the PCR, a 108-bp fragment was found to be amplified with sense primer 49 and antisense primer 51. Using both total RNA from xylem and genomic DNA of poplar, as a template, the same fragment was amplified. The PCR product was subcloned as a

blunt-end fragment in the SmaI site of pGem2 yielding plasmid pPLC1. The nucleotide and deduced amino acid sequences are shown in Figure 1 (positions 661-768).

To isolate a full-length cDNA, the 108-bp fragment was used as a probe to screen a leaf cDNA library from poplar. Three different clones, designated pPLC2, pPLC3, and pPLC4 were identified out of 80,000 clones. The nucleotide sequence of pPLC4 and the deduced amino acid sequence are shown in Figure 1. The cDNA insert is 1375 nucleotides in length and contains one open reading-frame of 1092 nucleotides encoding a protein of 364 amino acids (calculated M 39,720; Pi 5.45). The pPCL2 clone contains a cDNA of 1,420 nucleotides and contains also one ORF of 1,092 nucleotides encoding a protein of 364 amino acids. There is only a 3-amino acid difference between the proteins of the pPCL4 and the pPCL2 clones. Amino acids 97, 191, and 361 in the protein of pPCL2 are leucine, isoleucine, and phenylalanine, respectively. Thus a mixture of at least three closely related isoforms, as shown by the two isoforms present in peptide 45 and by the amino acid sequence of peptide 51 which is entirely found back in the deduced OMT sequence.

EXAMPLE 4

Analysis of the transgenic poplar trees for a modified lignin content

A. Transformation of poplar

With the poplar OMT cDNA clone (pPCL4) five different sense and antisense constructs were made resulting in five plasmids: p35SSOM3A (a 500 bp 5'end fragment in sense), p35SASOM3A (a 500 bp 5' end fragment in antisense); p35SSOM3B (a 900 pb 3'

end fragment in sense), p35SASOM3B (a 900 pb 3' end fragment in antisense) and p35SASOM3C (the full length OMT clone in antisense). All the constructs are under the control of the constitutive 35S Cauliflower Mosaic Virus (CaMV) promoter. These constructs were introduced in poplar via an 'Agrobacterium tumefaciens' - mediated transformation of stem explants.

For the plasmids p35SSOM3A (SA), p35SASOM3A (ASA), p35SSOM3B (SB) and p35SASOM3B (ASB) 16,21,7 and 13 independent transformants were regenerated, respectively. Control transformations were performed using plasmid pGSJ780A (this plasmid contains only the 35S CaMV promoter).

2. Estimation of the OMT antisense RNA amount in leaves from ASA and ASB plants.

Using single stranded (ribo) probes (which only hybridise with the antisense RNA) RNA gel blots were performed. The antisense B RNA was detected (strongly) in candidate ASB 5B and candidates ASB 3Am ASB 5A and ASB 7A.

The antisense A RNA levels were high in candidates ASA 1A and candidates ASA 5B, ASA 17A, ASA 6A and ASA 2B. No antisense RNA was detected in transgenic plants containing the pGSJ780A T-DNA. The difference in the antisense RNA levels can be explained by position effects.

3. Steady-state OMT sense RNA in leaves from SA and SB plants.

In an analogous way, by performing Northern blots with single stranded riboprobes which only detect sense RNA, SA and SB plants were analysed.

The sense B RNA can be detected easily in candidates SB 5A and SB 8A and not in candidates SB

2B, SB 4A, SB 7A and SB 10A.

The sense A RNA amount is high in candidates SA 11A and SA 12A, low in candidates SA 9A, SA 18A and SA 23A.

In all the transformants the endogenous OMT mRNA could not be detected (this is in agreement with the fact that the OMT is strongly expressed in xylem and very weak in leaves). All these different transgenics have the same phenotype as the wild type poplar and control plants.

4. OMT activity towards caffeic acid in the transgenic plants.

The OMT activity was measured in different organelles/tissues (Tables 3 and 4). Two candidates (p35SASOM3B 4A and 6A) with a lower OMT activity in petioles, xylem and phloem (two to three times lower) in comparison to wild type and control plants, were identified.

For both these candidates, there was no correlation between the reduced OMT activity and the amount of antisense RNA present in these plants.

5. Lignin analysis of the transgenic poplars.

The transgenic plants were analysed for their lignin composition (Table 5). Lignin is a complex polymer of three different units: p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomers. Poplar lignin contains both guaiacyl and syringyl units. A typical syringyl/guaiacyl (S/G) ratio for poplar is 2/1. Due to the antisense inhibition we expect that the monomer ratio will be modified, resulting in a lower S/G ratio. Lignin characterisation of xylem was performed by the analysis of degradation products recovered from

thioacidolysis. This method allows the estimation of the different units, involved in the lignin characteristic structure.

For the wild type and control plants a typical S/G ratio was found (2/1, Table 5). However, the two candidates ASB 4A and ASB 6A have a lower S/G ratio, 1.47 and 1.43, respectively. Like the bm3 maize mutants (these mutants have a lower S/G ratio (.039) in comparison with wild type maize (1.72) we notice that the S/G ratio of the two ASB poplars has been modified (although not as drastically as was the case for the maize mutants).

TABLE 3
OMT activity in petioles of the different ASB, ASA, SB and SA plants (in cpm).

Sample		Replicates 1	2	3	Mean
ASB	6A	-	2460	1252	1856
	8A	6074	5710	6931	6238
	4A	1097	1946	711	1251
	5B	2419	6295	5764	4826
ASA	23A	1954	4342	3834	3376
	17A	5001	7596	5386	5994
	2B	2602	3926	5796	4108
SB	4A	2527	5799	5226	4517
	10A	2252	4970.	5631	4284
	5A	4726	4672	4838	4755
	7A	2686	6302	7490	5492
SA	13A	3638	5423	3697	4252
	3B	3269	4201	6875	4781
	2B	2479	6626	5842	4982
	7A	5338	8244	8691	7424
WT	T1	3231	2843	7577	4550
	T2	3996	6171	6433	5533
	T3	3470	5152	10028	6216

WT = Control Plants.

TABLE 4

OMT activity in xylem and phloem of the candidates ASB 4A and ASB 6A (in cpm). Mean value, last lane.

Sample	Replicates 1	2	3	Mean
ASB 4AX	2465	1624	743	1610
F	3530	1108	1482	2040
ASB 6AX	3089	1845	1152	2028
F	4258	2063	3143	3154
WT T1X	5040	4440	2262	3914
F	8665	5505	8809	7659

WT = Control plants

TABLE 5

Syringyl/guaiacyl ratio of the different ASB, ASA, SB and SA plants.

Sample		Replicates 1	2	Mean
ASB	18A	2.17	2.41	2.29
	5A	1.61	1.84	1.73
	4A	1.34	1.60	1.47
	6A	1.28	1.59	1.43
ASA	7B	1.93	2.48	2.20
	15A	1.80	2.35	2.07
	23A	1.88	2.13	2.00
SB	4A	1.95	2.09	2.02
SA	1B	2.07	2.20	2.14
WT	T1	2.02	1.98	2.00

WT = Control Plants

EXAMPLE 5

Isolation of a tobacco OMT1 clones pTOMT1A and pTOMT1B.

Using similar procedures described for the isolation of the poplar OMT cDNA, a tobacco OMT1 cDNA clones were isolated from a leaf cDNA library prepared from RNA of TMV infected tobacco leaves. These clones were isolated by PCR using sequence information of the purified tobacco OMT1 protein. The combined sequence of two clones covering the complete coding sequence of tobacco OMT1 are shown in Figure 2. The underlined part represent sequences found in clone A. the dotted part represents sequences found in clone B.

A complete cDNA clone of OMTI has been isolated from a stem cDNA tobacco library. Its sequence is shown in Figure 3.

EXAMPLE 6

Immunoscreening of a λgt11 expression library and characterisation of the MOT III cDNA clone o3.614

The λgt11 phages were plated on Escherichia coli Y1090 and fusion proteins induced with 10mM IPTG. The proteins bound to nitro-cellulose filters were screened by immunodetection using anti-tobacco OMT III polyclonal antibody (Hermann et al., 1987; Dumas, 1990). Three positive clones were purified after immunoscreening of the λgt11 library. The length of their cDNA inserts was determined by PCR. Aliquots of phage lysates (5μl) were heated for 5 mins at 94°C and used as template for PCR. The amplification mixture consists of 10mM Tri-HCl pH 8.3, 11mM KCl, 1.6mM $MgCl_2$, 1mM DTT, 200μM each dNTP, 1μM each oligonucleotide primer, and 1 unit of Taq polymerase (Beckman) in a

final volume of 50µl. The amplification program consisted of 25 cycles of denaturation (94°C, 1 min), annealing (40°C, 1 min), and primer extension (72°C, 1 min). The longest clone, named o3.614, was 614 bp long and was submitted to phage amplification and DNA purification by CsCl gradient (4). The 614 bp cDNA insert was subloned by EcoRI restriction digestion in pBluescript KS(+)(Stratagene, Inc). According to standard methods (4), deletion from both the extremities of the plasmid were generated by ExoIII-Mung Bean digestions and the primers and the T7 DNA polymerase.

EXAMPLE 7

Construction and screening of a λZapII cDNA library from tobacco leaf RNA and characterisation of a complete clone

Poly(A)+RNA from 48 hours TMV infected leaves was used to construct an oligo(dT) primed cDNA. Double-stranded cDNA was ligated to hemi-phosphorylated EcoR I/Not I adaptors (Pharmacia), ligated into λZapII vector (Stratagene, Inc) and packaged using Gigapack in vitro packaging extracts (Stratagene, Inc). The resulting cDNA library titled 1.8 X 10^7 pfu/µg cDNA. The o3.614 clone was used as DNA probe. The 614 bp DNA fragment was purified from purified from 0.8% agarose gel (PrepAGene, BIORAD), 32p labelled by random oligonucleotide-primed synthesis and used to screen a λZapII library made by standard protocols (4). After three cycles of screening, twelve positive clones were isolated from approximately 1.8 X 10^5 plaque-forming units. cDNA inserts from positive phage were rescued as

Bluescript plasmids by R408 helper phage mediated in vivo excision, as described by the manufacture (Stratagene, Inc). Cloned insert DNA was isolated by Not I digestion and analysed on 1% agarose gels. The cDNA clones isolated from λZapII cDNA library were mapped with restriction endonucleases and then sequenced on both strands by the dideoxy chain termination method 95) using T7 DNA polymerase (Pharmacia). For sequencing the internal regions, synthetic oligonucleotide primers (18mers) were designed from the DNA sequence previously determined. The complete sequence of a OMT III cDNA clone is given in Figure 4. The amino acid sequences of peptides obtained by trypsic digestion of the purified OMT II and OMT III proteins all correspond to the aminoacid sequence derived from OMT III cDNA clones. These evidences suggest the occurrence of only one mRNA species for the "Pathogenesis-Related" forms OMT II and OMT III but of 2 forms for the lignification-related OMT I.

EXAMPLE 8

Localisation of OMT I transcripts by in situ hybridisation.

In petiole sections, OMT I mRNAs were localised in parenchyma cells of xylem and phloem, with a marked signal around the nuclei. In leaf sections, OMT I mRNAs were found to accumulate particularly in the upper and lower epidermis in a ring of tissue surrounding TMV-induced necrotic lesions, were no cell-type specific hybridisation was found in the healthy leaf.

EXAMPLE 9

Design of antisense vectors

Five different poplar OMT antisense constructs

have been made. A 500 bp BamHI fragment corresponding to the 5' end of the poplar OMT clone and a 900-bp BamHI fragment corresponding to the 3' end of the poplar OMT clone were cloned in the BamHI site of vector pGSJ780A, in both sense and antisense direction yielding the four plasmids p35SSOM3A, p35SASOM3A, p35SASOM3A, p35SSOM3B, and p35SASOM3B (Figure 5). All OMT fragments are under the control of the CaMV 35S promoter. The full-length OMT clone was cloned in the vector pGSJ780A in antisense direction by PCR yielding plasmid p35SASOM3C (Figure 6). The correct direction of the inserts has been confirmed by sequencing. The vector pGSJ780A is a binary vector with the pVS1 origin of replication and a Sm/Sp resistance gene for selection in Agrobacterium tumefaciens. Between the T-DNA borders there is a nos-nptII-ocs cassette and a multiple cloning site).

The construction of the tobacco antisense vector follows that described for the poplar vectors. The insert was inserted into the vector pGSJ780A.

CLAIMS

1. A DNA encoding caffeic acid O-methyl transferase contained in the clones pPLC4 and pTOMTI and variants thereof such as are permitted by the degeneracy of the genetic code or the functional equivalents thereof.

2. A recombinant DNA comprising the DNA claimed in claim 1 under control of a transcriptional control sequence operative in plants.

3. A recombinant DNA as claimed in claim 2, for the down-regulation of lignin biosynthesis in which the said DNA is in antisense orientation.

4. A recombinant DNA as claimed in claim 2, for the amplification of lignin biosynthesis, in which the said DNA is in sense orientation.

5. A DNA comprising a transcriptional initiation region operative in plants operatively linked to a DNA sequence encoding RNA complementary to a substantial number of bases showing substantial homology to an mRNA encoding the protein produced by the gene in pPLC4 or pTOMT1 so as to initiate production of mRNA therefrom.

6. A DNA having the nucleotide sequence given in Figure 1.

7. A DNA having the nucleotide sequence given in Figure 2.

8. A DNA having the nucleotide sequence given in Figure 3.

9. A DNA having the nucleotide sequence given in Figure 4.

10. A plant cell, and a plant derived therefrom having stably incorporated in its genome by transformation a DNA as claimed in any one of claims 1 to 9 in sense or antisense orientation, and the fruit and seeds of said plants.

11. A method for the regulation of lignin biosynthesis in a plant comprising stably incorporating into the genome of a plant by transformation a recombinant DNA encoding the enzyme caffeic acid O-methyl-transferase in sense or antisense orientation under control of a transcriptional initiation region operative in plants.

FIG.1

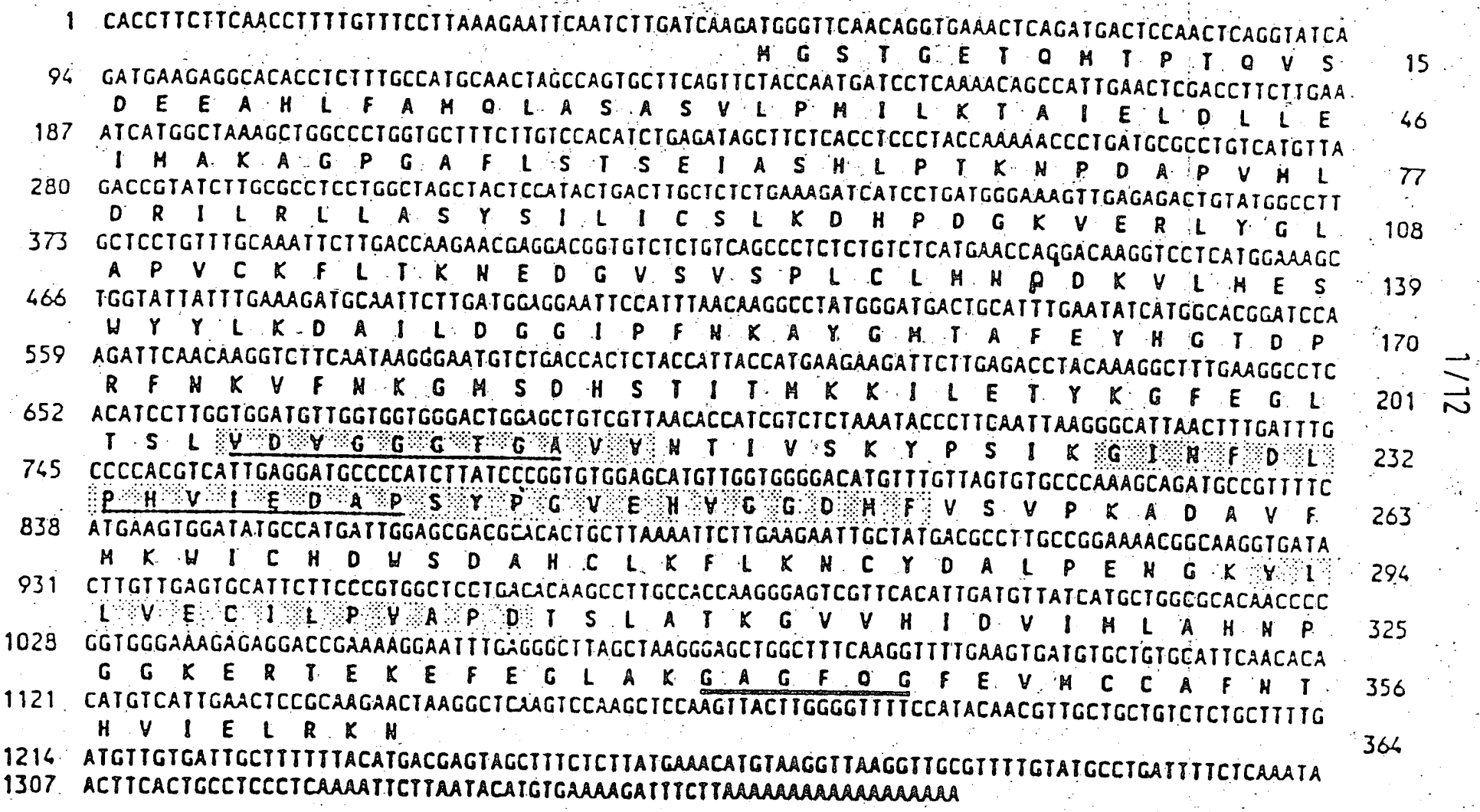
1 CACCTTCTTCAACCTTTTGTTTCCTTAAAGAATTCAATCTTGATCAAGATGGGTTCAACAGGTGAAACTCAGATGACTCCAACTCAGGTATCA
H G S T G E T Q M T P T Q V S 15
94 GATGAAGAGGCACACCTCTTTGCCATGCAACTAGCCAGTGCTTCAGTTCTACCAATGATCCTCAAAACAGCCATTGAACTCGACCTTCTTGAA
D E E A H L F A M Q L A S A S V L P M I L K T A I E L D L L E 46
187 ATCATGGCTAAAGCTGGCCCTGGTGCTTTCTTGTCCACATCTGAGATAGCTTCTCACCTCCCTACCAAAAACCCTGATGCGCCTGTCATGTTA
I M A K A G P G A F L S T S E I A S H L P T K N P D A P V M L 77
280 GACCGTATCTTGCGCCTCCTGGCTAGCTACTCCATACTGACTTGCTCTCTGAAAGATCATCCTGATGGGAAAGTTGAGAGACTGTATGGCCTT
D R I L R L L A S Y S I L I C S L K D H P D G K V E R L Y G L 108
373 GCTCCTGTTTGCAAATTCTTGACCAAGAACGAGGACGGTGTCTCTGTCAGCCCTCTCTGTCTCATGAACCAGGACAAGGTCCTCATGGAAAGC
A P V C K F L T K N E D G V S V S P L C L H N P D K V L H E S 139
466 TGGTATTATTTGAAAGATGCAATTCTTGATGGAGGAATTCCATTTAACAAGGCCTATGGGATGACTGCATTTGAATATCATGGCACGGATCCA
W Y Y L K D A I L D G G I P F N K A Y G M T A F E Y H G T D P 170
559 AGATTCAACAAGGTCTTCAATAAGGGAATGTCTGACCACTCTACCATTACCATGAAGAAGATTCTTGAGACCTACAAAGGCTTTGAAGGCCTC
R F N K V F N K G M S D H S T I T M K K I L E T Y K G F E G L 201
652 ACATCCTTGGTGGATGTTGGTGGTGGGACTGGAGCTGTCGTTAACACCATCGTCTCTAAATACCCTTCAATTAAGGGCATTAACTTTGATTTG
T S L V D V G G G T G A V V N T I V S K Y P S I K G I N F D L 232
745 CCCCACGTCATTGAGGATGCCCCATCTTATCCCGGTGTGGAGCATGTTGGTGGGGACATGTTTGTTAGTGTGCCCAAAGCAGATGCCGTTTTC
P H V I E D A P S Y P G V E H V G G D M F V S V P K A D A V F 263
838 ATGAAGTGGATATGCCATGATTGGAGCGACGCACACTGCTTAAAATTCTTGAAGAATTGCTATGACGCCTTGCCGGAAAACGGCAAGGTGATA
M K W I C H D W S D A H C L K F L K N C Y D A L P E N G K V I 294
931 CTTGTTGAGTGCATTCTTCCCGTGGCTCCTGACACAAGCCTTGCCACCAAGGGAGTCGTTCACATTGATGTTATCATGCTGGCGCACAACCCC
L V E C I L P V A P D T S L A T K G V V H I D V I M L A M N P 325
1028 GGTGGGAAAGAGAGGACCGAAAAGGAATTTGAGGGCTTAGCTAAGGGAGCTGGCTTTCAAGGTTTTGAAGTGATGTGCTGTGCATTCAACACA
G G K E R T E K E F E G L A K G A G F Q G F E V M C C A F N T 356
1121 CATGTCATTGAACTCCGCAAGAACTAAGGCTCAAGTCCAAGCTCCAAGTTACTTGGGGTTTTCCATACAACGTTGCTGCTGTCTCTGCTTTTG
H V I E L R K N 364
1214 ATGTTGTGATTGCTTTTTTACATGACGAGTAGCTTTCTCTTATGAAACATGTAAGGTTAAGGTTGCGTTTTGTATGCCTGATTTTCTCAAATA
1307 ACTTCACTGCCTCCCTCAAAATTCTTAATACATGTGAAAAGATTTCTTAAAAAAAAAAAAAAAAAA

FIG. 2 (1/2)

```
   1  GCCGGCCCGG GCGCGGCGAT TTCTCCTTCT GAATTAGCTG CTCAGCTCTC
  51  AACCCAGAAC CCAGAAGCAC CCGTTATGCT TGATCGGATG CTTAGGCTAC
 101  TTGCTACTTA CTCTGTTCTC AATTGTACTC TTAGAACACT GTCTGATGGC
 151  AGTGTTGAGA GGCTTTATAG TCTGGCTCCG GTTTGTAAGT TCTTGACTAA
 201  GAATGCTGAT GGTGTTTCTG TTGCCCCACT TTTGCTTATG AATCAAGATA
 251  AAGTTCTTAT GGAGAGCTGG TACCACTTAA AAGATGCAGT ACTAGATGGT
 301  GGAATCCCAT TCAACAAGGC CTATGGAATG ACAGCATTTG AGTACCATGG
 351  CACAGATCCA AGATTCAACA AAGTTTTCAA CCGTGGAATG TCTGATCACT
 401  CCACTATGTC AATGAAAAAG ATTCTTGAGG ACTACAAAGG ATTTGAAGGC
 451  CTAAATTCCA TTGTCGATGT TGGTGGTGGA ACTGGCGCTA CTGTTAACAT
 501  GATTGTCTCC AAACATCCCT CTATTAAGGG TATTAACTTT GATTTACCAC
 551  ATGTTATTGG AGATGCTCCA GCTTACCCTG GTGTCGAGCA CGTTGGTGGC
 601  GACATGTTTG CCAGTGTGCC AAAAGCAGAT GCCATTTTCA TGAAGTGGAT
```

651 TTGTCATGAT TGGAGCGACG AGCATTGCCT AAAATTCTTG AAGAATTGCT

701 ATGAAGCACT ACCTGCAAAT GGGAAGGTGA TAATAGCGGA GTGCATACTT

751 CCAGAGGCCC CAGATACATC ACTTGCAACT AAGAATACAG TACATGTTGA

801 TATTGTGATG TTAGCACATA ACCCAGGAGG CAAAGAAAGG ACTGAGAAGG

851 AATTTGAGGC TTTGGCTAAG GGCGCTGGTT TTACTGGATT CGCAAGGCTT

901 GTTGCGCTTA CAACACTTGG GTCATGGAAT TCAACAAATA ATTAATCGAT

951 TCCTTTGGAG AATTAAGCAA TATACTGTTC ATTTTGCATT TTGAAATTCT

1001 ACTTTTCACA GAGTGGCTTT ACTGCGAAAT AAAAGAAATA TATAGCTTTT

1051 ACCTTGAAAA GATCAATGTT CAAAGGGAAA AAAAAAAGGA AGATGAAATA

1101 ATTGCTCTCA GAAAAGCAGT GTGTTAGGAA AAAGCTTTTT AGCTGGATTT

1151 TGAATTTTTA TTGTATGTAT TTCTGTAATA CACATGTATT GAAGGAATAC

1201 TAGTTTTCGA CCAATCATAT TTCTTTGAAA AAAAAAAAAA AAAA

FIG.2(2/2) OMT I Sequences : plasmid A + plasmid B

———— : A

--------- : B

FIG. 3 (1/2)

```
ATTCCTTCAACTTACCCAATTAAGTCATCGAAAAATCTGAAACAGAACTAAAAGTAAAAT
1
                                                              M
TTATTTGCCATGCAATTGTGTAGTGCTTCTGTACTTCCTATGGTCCTAAAATCAGCCGTAGA
119
 L  F  A  M  Q  L  C  S  A  S  V  L  P  M  V  L  K  S  A  V  E
21
GAATTAGCTGCTCAGCTCTCAACTCAGAACCCAGAAGCACCTGTTATGCTTGATCGGATGCT
239
 E  L  A  A  Q  L  S  T  Q  N  P  E  A  P  V  M  L  D  R  M  L
61
AGTGTTGAGAGGCTTTATAGTCTGGCTCCCGTCTGTAAGTACTTGACTAAGAATGCTGATGG
359
 S  V  E  R  L  Y  S  L  A  P  V  C  K  Y  L  T  K  N  A  D  G
101
TACCACTTAAAAGATGCAGTACTAGATGGCGGAATCCCATTCAACAAAGCCTATGGAATGCA
419
 Y  H  L  K  D  A  V  L  D  G  G  I  P  F  N  K  A  Y  G  M  T
141
TCTGATCACTCCACTATGTCAATGAAGAAGATTCTTGAGGACTACAAAGGATTTGAAGGCCT
599
 S  D  H  S  T  M  S  M  K  K  I  L  E  D  Y  K  G  F  E  G  L
181
AAATATCCCTCTATTAAGGGCATTAACTTTGATTTGCCACATGTAATTGGAGATGCTCCAAC
719
 K  Y  P  S  I  K  G  I  N  F  D  L  P  H  V  I  G  D  A  P  T
221
GCCATTTTCATGAAGTGGATTTGTCATGATTGGAGCGATGAGCATTGCCTAAAATTCTTGAA
839
 A  I  F  M  K  W  I  C  H  D  W  S  D  E  H  C  L  K  F  L  K
261
CCAGAGGCCCCAGATACATCACTTGCAACTAAGAATACAGTACATGTTGATATTGTTATGTT
959
 P  E  A  P  D  T  S  L  A  T  K  N  T  V  H  V  D  I  V  M  L
301
GGCGCTGGTTTTACTGGATTCGCAAGGCTTGTTGCGCTTACAACACTTGGGTCATGGAATTC
179
 G  A  G  F  T  G  F  A  R  L  V  A  L  T  T  L  G  S  W  N  S
341
CATTTGGAAATTCTACTTTTCTCAGAGTGGCTTGACTGTGAAATAAAAGAAATATAGCTTTT
1199
AAAGCAATGTGTTAGGAAAAAGCTTTTTTAGCTGGATTTTGAATTTTACTGTATGTATTTCT
1319
```

FIG. 3 (2/2)

```
GGGTTCAACAAGCGAGAGCCAGAGTAACAGTCTCACTCACACAGAAGACGAAGCTTTC
                                                          118
  G  S  T  S  E  S  Q  S  N  S  L  T  H  T  E  D  E  A  F
                                                           20
ACTTGACCTTCTTGAGCTAATGGCTAAGGCTGGTCCAGGTGCAGCTATTTCTCCTTCT
                                                          238
  L  D  L  L  E  L  M  A  K  A  G  P  G  A  A  I  S  P  S
                                                           60
TAGGCTACTTGCTTCTTACTCTGTTCTCAATTGTACTCTTAGAACACTGCCTGATAGC
                                                          358
  R  L  L  A  S  Y  S  V  L  N  C  T  L  R  T  L  P  D  S
                                                          100
TGTTTCTGTTGCCCCACTTTTGCTTATGAATCAAGATAAAGTTCTTATGGAGAGCTGG
                                                          418
  V  S  V  A  P  L  L  L  M  N  Q  D  K  V  L  M  E  S  W
                                                          140
AGCATTTGAGTACCATGGCACAGATCCAAGATTCAACAAAGTGTTCAACCGTGGAATG
                                                          598
  A  F  E  Y  H  G  T  D  P  R  F  N  K  V  F  N  R  G  M
                                                          180
AAATTCCATTGTTGATGTTGGTGGTGGAACGGGTGCTACTGTTAACATGATTGTCTCT
                                                          718
  N  S  I  V  D  V  G  G  G  T  G  A  T  V  N  M  I  V  S
                                                          220
TTACCCCGGTGTCGAGCACGTTGGTGGCGACATGTTTGCTAGTGTGCCAAAAGCAGAT
                                                          838
  Y  P  G  V  E  H  V  G  G  D  M  F  A  S  V  P  K  A  D
                                                          260
GAATTGCTATGAAGCACTACCTGCAAATGGGAAGGTGATAATTGCAGAGTGCATACTT
                                                          958
  N  C  Y  E  A  L  P  A  N  G  K  V  I  I  A  E  C  I  L
                                                          300
AGCACATAACCCAGGAGGCAAAGAAAGGACTGAGAAGGAATTTGAGGCTTTGGCTAAG
                                                         1078
  A  H  N  P  G  G  K  E  R  T  E  K  E  F  E  A  L  A  K
                                                          340
AACAAGTAATTAATCGATTCCTTAATTTGAAGGATTAAGCAATATACTGTTCGTTTTG
                                                         1198
  T  S  N  *
                                                          364
AACTTGAAAAGATTGATGTTCAAAAGAAAAAAAGGAAGATGAAATAATTGCTCTCAGA
                                                         1318
GTTATACACATGTATTGAAGGAATACTAGTTTTCGACCAAAAAAAAAAAAA
                                                         1431
```

FIG. 4 (1/5) OMT III CDNA isolated from the λ Zap II library of tobacco leaf

```
1   AACAAAAACACTCTAAAAGGAAAAGACTAGGAGAATAATTACACTACAACTCTTATAGCT

61  AATTCTTGTCTCAAGATTTTCACCTATGGAATCCTCAACCAAAAGCCAAATACCAACACA

                          M  E  S  S  T  K  S  Q  I  P  T  Q   12

121 ATCAGAAGAAGAGCGTAACTGCACATATGCCATGCAACTATTGTCATCTTCAGTCCTCCC

    S  E  E  E  R  N  C  T  Y  A  M  Q  L  L  S  S  S  V  L  P   32

181 CTTTGTGTTGCATTCAACAATTCAATTGGAAGTTTTTGAGATATTAGCCAAATCTAATGA

    F  V  L  H  S  T  I  Q  L  E  V  F  E  I  L  A  K  S  N  D   52

241 CACTAAACTTTCTGCTTCTCAAATTGTTTCTCAAATTCCTAACTGCAAGAATCCTGATGC

    T  K  L  S  A  S  Q  I  V  S  Q  I  P  N  C  K  N  P  D  A   72
```

FIG. 4 (2/5)

```
301 AGCTACTATGTTAGATAGGATGCTTTATGTGTTGGCTAGTTACTCGTTGTTTACTTGTTC
      A   T   M   L   D   R   M   L   Y   V   L   A   S   Y   S   L   F   T   C   S   92
361 CATTGTTGAGGATGAAGAAAATAATGGGGGCCAGAAAAGAGTGTATGGTTTGTCACAAGT
      I   V   E   D   E   E   N   N   G   G   Q   K   R   V   Y   G   L   S   Q   V   112
421 GGGAAAATTCTTTGTTAGAGATGAAGATGGTGCATCAATGGGGCCACTTTTGGCTTTGCT
      G   K   F   F   V   R   D   E   D   G   A   S   M   G   P   L   L   A   L   L   132
481 TCAAGATAAAGTATTCATAAACAGCTGGTTTGAACTAAAAGATGCAGTTCTTGAAGGAGG
      Q   D   K   V   F   I   N   S   W   F   E   L   K   D   A   V   L   E   G   G   152
541 AGTTCCATTTGACAGGGTACACGGTGTTGTCCATGCATTTGAATATCCAAAATCGGACCC
      V   P   F   D   R   V   H   G   V   V   H   A   F   E   Y   P   K   S   D   P   172
```

FIG. 4 (3/5)

```
601 AAAATTCAATGATGTTTTCAACAAGGCAATGATCAATCACACAACTGTAGTCATGAAAAA

    K  F  N  D  V  F  N  K  A  M  I  N  H  T  T  V  V  M  K  K  192

661 AATACTTGAAAATTACAAAGGTTTTGAGAACCTTAAAACTTTGGTTGATGTTGGAGGTGG

    I  L  E  N  Y  K  G  F  E  N  L  K  T  L  V  D  V  G  G  G  212

721 TCTTGGAGTTAACCTCAAGATGATTACATCTAAATACCCCACAATTAAGGGCACTAATTT

    L  G  V  N  L  K  M  I  T  S  K  Y  P  T  I  K  G  T  N  F  232

781 TGATTTGCCACATGTTGTTCAACATGCCCCTTCCTATCCTGGGGTGGAACATGTTGGGGG

    D  L  P  H  V  V  Q  H  A  P  S  Y  P  G  V  E  H  V  G  G  252

841 AGATATGTTTGAAAGTGTTCCAGAAGGAGATGCTATTTTTATGAAGTGGATTCTTCATGA

    D  M  P  F  S  V  P  E  G  D  A  I  F  M  K  W  I  L  H  D  272
```

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FIG. 4 (4/5)

```
901  CTGGAGTGATAGTCACAACCTCAAGTTGCTAAAGAACTGCTACAAGGCTCTACCAGACAA

       W  S  D  S  H  N  L  K  L  L  K  N  C  Y  K  A  L  P  D  N  292

961  TGGAAAGGTGATTGTTGTTGAGGCCATTTTACCAGTGAAACCAGACATTGACACCGCAGT

       G  K  V  I  V  V  E  A  I  L  P  V  K  P  D  I  D  T  A  V  312

1021 GGTTGGCGTTTCGCAATGTGATTTGATCATGATGGCTCAAAATCCTGGAGGCAAAGAGCG

       V  G  V  S  Q  C  D  L  I  M  M  A  Q  N  P  G  G  K  E  R  332

1080 ATCGGAAGAGGAGTTTCGAGCCTTGGCTACTGAAGCTGGATTCAAAGGCGTTAACTTAAT

       S  E  E  E  F  R  A  L  A  T  E  A  G  F  K  G  V  N  L  I  352

1141 ATGTTGTGTCTGTAATTTTTGGGTCATGGAATTCTGCAAGTAGATTTCTACTGTACATTG
```

C C V C F P W V M E F C K *

1201 AGTTTCTACTACTCTTGAGTATCCATTTATGGCAATCTGGGACTGGAATTGCAGCTTAGT

1261 CCAGATTGAACATTGATATTCCTAATAATATTTCTATTATTTCCCTTGTTTATTTCTCTT

1321 GTATGAAAGGATGTCATTTTGAGTATTGATAATCATGTTCTCTAGGACAGAAATTGTAAC

1381 TTTGTCCAACTTTATTGATATTCCTAGTAAGATTTATATGACATGTGTCTCTGGTTTGAG

1441 AAGAGTTTCAATATCTAAAAAAAAAAAAAAA

FIG. 4 (5/5)

FIG. 5

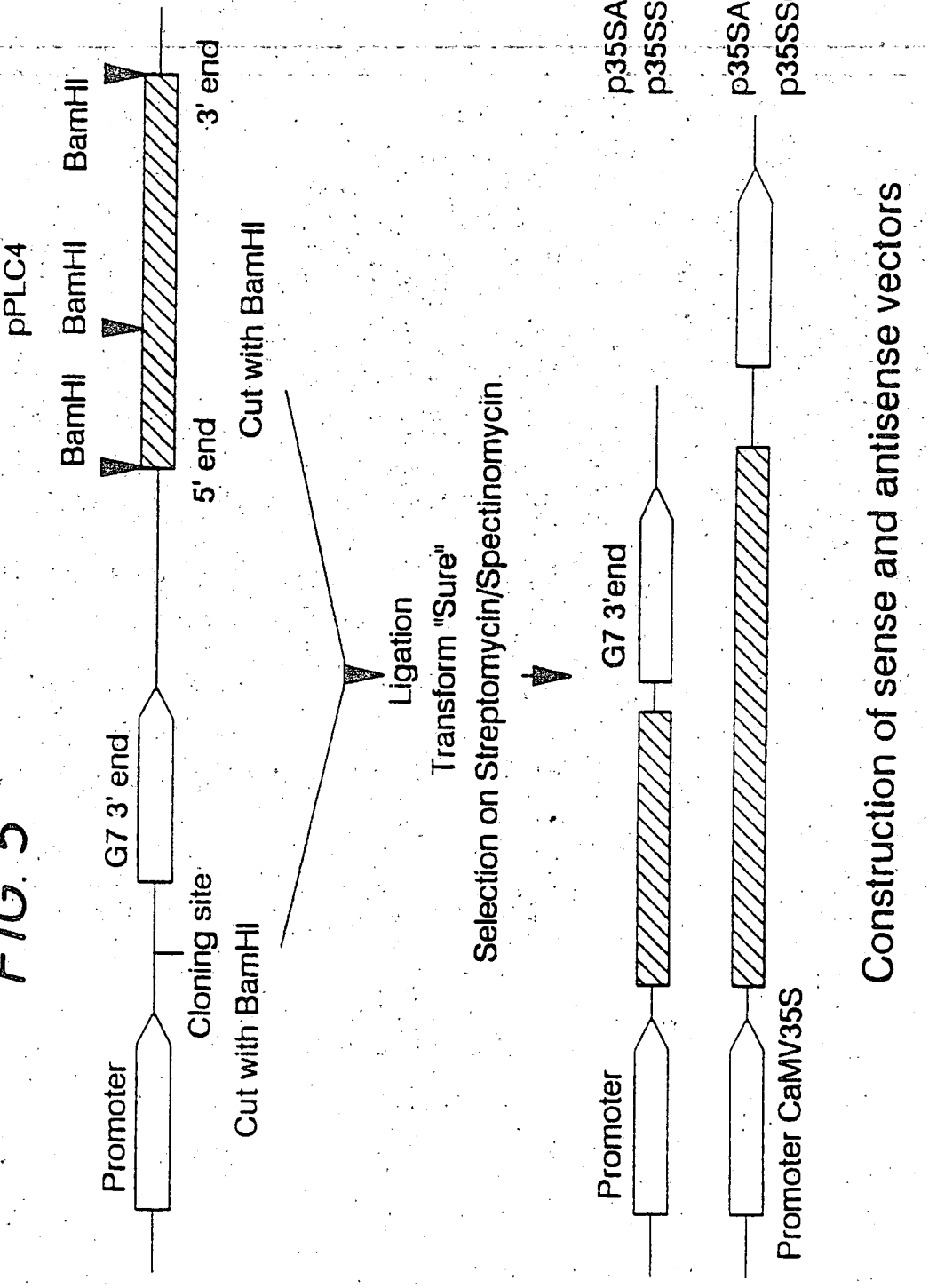
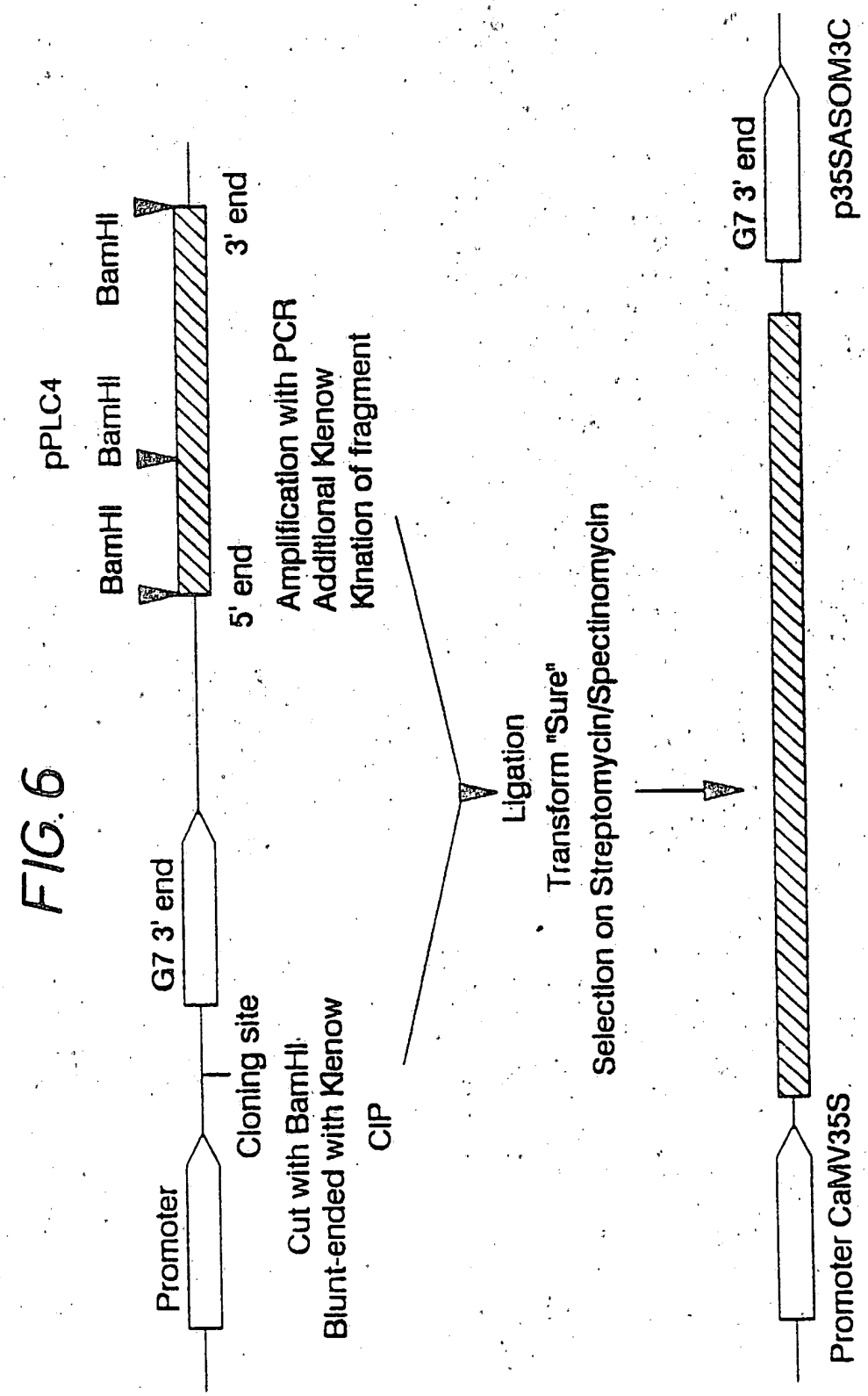

pPLC4
BamHI
BamHI
BamHI
3' end
5' end
G7 3' end
Cloning site
Promoter
Cut with BamHI
Cut with BamHI
Ligation
Transform "Sure"
Selection on Streptomycin/Spectinomycin
G7 3'end
Promoter
p35SASOM3A
p35SSOM3A
p35SASOM3B
p35SSOM3B
Promoter CaMV35S
Construction of sense and antisense vectors

FIG. 6


pPLC4
BamHI
BamHI
BamHI
3' end
5' end
Amplification with PCR
Additional Klenow
Kination of fragment
G7 3' end
Cloning site
Promoter
Cut with BamHI
Blunt-ended with Klenow
CIP
Ligation
Transform "Sure"
Selection on Streptomycin/Spectinomycin
G7 3' end
p35SASOM3C
Promoter CaMV35S

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01640

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/54; C12N15/82; C12N15/11; C12N5/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	PLANT PHYSIOLOGY. vol. 97, no. 1, September 1991, ROCKVILLE, MD, USA. pages 7 - 14 GOWRI, G., ET AL. 'Stress responses in Alfalfa (Medicago sativa L.)'	1
Y	see the whole document particularly page 13 right col. last para	2-11

X	J. CELL. BIOCHEM. SUPPL.,MEETING HELD JAN 10-17, 1991. vol. 15A, 1991, page 144 CAMPBELL, W.H., ET AL. 'Seasonal expression of a lignin specific O-methyltransferase cloned from aspen developing secondary xylem' see the whole document	1

	-/--	

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 DECEMBER 1992	16. 12. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PLANT PHYSIOLOGY, (SUPPL.) vol. 93, no. 1, May 1990, ROCKVILLE, MD, USA. page 15 BUGOS, R.C., ET AL. 'Cloning and expression of lignin bispecific O-methyltransferase' see abstract 83 ---	1
Y	CA,A,2 005 597 (SCHUCH) 15 June 1990 see page 13, line 14 - line 34 ---	2-5,10, 11
Y	BIOLOGICAL ABSTRACTS vol. 73 , 1982, Philadelphia, PA, US; abstract no. 64323, MARIGO, G., ET AL. 'Biosynthesis of carbon-14-labeled ferulic acid and sinapic acid' see abstract & J. LABELLED COMPD. RADIOPHARM. vol. 18, no. 5, 1981, pages 695 - 702 ---	6
Y	BIOLOGICAL ABSTRACTS vol. 84 , 1987, Philadelphia, PA, US; abstract no. 19249, HERMANN, C., ET AL. 'Enzymatic synthesis of lignin: Purification to homogeneity of the three O-methyltransferases of tobacco and production of specific antibodies' see abstract & ARCH. BIOCHEM. BIOPHYS. vol. 253, no. 2, 1987, pages 367 - 376 ---	7-9
P,X	PLANT PHYSIOLOGY. vol. 98, no. 2, February 1992, ROCKVILLE, MD, USA. pages 796 - 797 DUMAS, B., ET AL. 'Nucleotide sequence of a complementary DNA encoding O-methyltransferase from poplar' see the whole document --- -/--	6

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	BIOLOGICAL ABSTRACTS vol. 94 , 1992, Philadelphia, PA, US; abstract no. 89217, JAECK, E., ET AL. 'Regulation of enzymes involved in lignin biosynthesis: Induction of O-methyltransferase mRNAs during the hypersensitive reaction of tobacco to tobacco mosaic virus' see abstract & MOL PLANT-MICROBE INTERACT. vol. 5, no. 4, 1992, pages 294 - 300	7,8
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O,P,X	PLANT PHYSIOLOGY, (SUPPL.) vol. 99, no. 1, May 1992, ROCKVILLE, MD, USA. page 19 PODILA, G.K., ET AL. 'Antisense expression of an aspen O-methyltransferase construct in transgenic tobacco via Agrobacterium' see abstract 110	2,3,5,10,11
A	PLANT PHYSIOLOGY. vol. 96, no. 2, June 1991, ROCKVILLE, MD, USA. pages 577 - 583 LAGRIMINI, L.M., ET AL. 'Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase' see the whole document	4,10,11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201640 SA 64222

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA-A-2005597	15-06-90	None	

CORRECTED VERSION *

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(81) Designated States: AU, BB, BG, BR, CA, CS, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, PL, RO, RU, SD, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: MODIFICATION OF LIGNIN SYNTHESIS IN PLANTS

(57) Abstract

The biosynthesis of lignin in plants is regulated by insertion into the plant genome by altering the plant's ability to synthesize the enzyme O-methyl-transferase, an enzyme involved in the lignin biosynthetic pathway. Production of O-methyl-transferase may be enhanced by insertion into the plant genome by transformation of one or more additional copies of the O-methyl-transferase gene or production may be inhibited by insertion of a gene encoding anti-sense mRNA directed against the mRNA encoded by the endogenous O-methyl-transferase gene.

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MODIFICATION OF LIGNIN SYNTHESIS IN PLANTS

This invention relates to the improvement of plants by the modification of lignin biosynthesis, particularly, but not exclusively, the improvement of digestibility of fodder crops.

Grassland farmers, and farmers of other fodder crops, face a difficult decision each year about when to cut their crops for conservation. All grass varieties of agricultural importance suffer from the disadvantage that during the normal increase in dry matter yield with growth, the digestibility decreases. The farmer, therefore, has, to compromise between a lower yield of highly digestible material and a higher yield of less digestible material. Another limitation is that harvesting at optimum maturity may be prevented by unfavourable weather. If the decline in digestibility could be controlled or delayed, higher yields of highly digestible material could be obtained and the prevailing weather conditions would not play such a major role in determining the quality of the harvested crop.

Digestibility of fodder crops is determined, among other factors, by the amount and quality of lignin deposition which has taken place during growth of the plants and the degree of secondary modification of lignin deposited. Beside cellulose and other poly- saccharides, lignins are an

essential component of cell wall in tissues like the sclerenchyma and the xylem of vascular plants. They play an important role in the conducting function of the xylem by reducing the permeability of the cell wall to water. They are also responsible for the rigidity of the cell wall, and, in woody tissues, they act as a bonding agent between cells, imparting to the plant a resistance towards impact, compression and bending. Finally, they are involved in mechanisms of resistance to pathogens by impeding the penetration or the propagation of the pathogenic agent.

Lignins are not only important in the productivity and performance of field crops but are also of great importance in trees for paper making. Considerable energy and chemical input is required to loosen, dissolve and remove lignin from the cellulose fibre which is required for paper making. In addition to these instances in which lignins present a constraint on the use of crop plants, lignins are also used as feedstocks for the preparation of speciality chemicals such as phenolics which can be used as precursors in chemical synthesis. Thus lignins and their biological and chemical modification are important.

It is one of the objects of the present invention to provide a biotechnological procedure for the modification both lignin content and lignin composition in plants.

Lignins are the product of a dehydrogenative polymerisation of three primary precursors: the trans-coniferyl, trans-sinapyl and trans-p-coumaryl alcohols. The monomers can occur in lignins in different proportions and with different types of

links both with each other and with the surrounding cell wall polysaccharides, thus producing a wide variety of polymers. These polymers, or "lignin cores" are always associated covalently with hemicelluloses. Most lignins also contain varying amounts of aromatic carboxylic acids in ester-like combinations. Such differences in the structure of lignins are usually found in plant species. However, differences in the composition of lignins, and even in the binding to the primary and secondary cell walls, can also occur in the same plant, between different tissues of different ages. The biosynthesis of lignin monomers (monolignols) is a part of the phenylpropanoid biosynthesis pathway, which is also responsible for the production of a wide range of compounds including flavonoid pigments, isoflavonoids, coumarin phytoalexins and cell division promoting dehydrodiconiferyl glucosides.

Phenylalanine is deaminated to produce cinnamic acid. This acid is then transformed by hydroxylation and methylation reactions, thus producing different acids substituted on the aromatic ring. The enzyme catalysing the methylation steps is O-methyl transferase (OMT). O-methyltransferases (S-adenosyl-L-methionine: O-methyltransferases; EC 2.1.1.6) thus play an important role in the biosynthesis of monolignols. By the O-methylation of caffeic acid and 5-hydroxyferulic acid, OMTs introduce one and two methoxy groups in the lignin monomers, respectively. The resulting two phenolics, ferulic acid and sinapic acid, respectively, are the precursors of coniferyl alcohol and sinapyl alcohol

which are together with coumaryl alcohol substrates for peroxidases (Lewis and Yamamoto, 1990).

The previous methylation reactions are also used in the synthesis of several other phenolic compounds. However, in those cells which are dedicated to the production of lignins such as vascular xylem cells of plants, the OMT plays a crucial role in the production of the phenolic precursors incorporated into the lignin polymer. The cinnamyl alcohols, synthesised in the cytoplasm, are then transported to the cell wall where they are polymerised by peroxidase in the presence of hydrogen peroxide.

When the surface growth of the cell ceases, it is followed by a phase of wall thickening (secondary wall formation). Lignification takes place during this phase. It starts in the cell corners and extends along the middle lamella, through the primary wall and, finally, to the secondary wall. External factors can induce qualitative and quantitative modifications in lignification. The synthesis of new types of lignins, sometimes in tissues which are not normally lignified, may be induced by infection with pathogenic microorganisms. Lignification is stimulated by light, as well as by low calcium levels, by boron, by mechanical stress and by infection.

As a first step in unravelling of lignin biosynthesis at the molecular level, we have undertaken the biochemical characteristics and cloning of O-methyltransferases (OMTs). Previously three different OMTs (OMT I, OMT II, and OMT III) have been purified from tobacco. OMT I used mainly

caffeic acid and 5-hydroxyferulic acid as a substrate and is the OMT actively present in healthy plants. OMT II and OMT III have a broader substrate specificity and also use catechol as substrate. Upon infection with TMV, an increase in activity of all three OMTs was shown. Based on this observation, it has been postulated that the OMT I is specifically involved in lignification, whereas OMT II and OMT III have a function in generating a lignin barrier upon infection. The importance of methylation in monolignol biosynthesis is well illustrated in brown-rib corn mutants. These plants exhibit a reduced lignin content and accumulate 5-hydroxyferulic acid) due to a low O-methyltransferase activity. Thus OMTs could be potential targets for modulation of lignification through the use of recombinant DNA technology.

Thus, plants with a reduced amount of lignin would be more efficiently used as a forage for cattle. The yield of milk and meat would be therefore increased. Furthermore, lignin may have a negative effect on plant growth. Thus, a reduction of the lignification in crops such as wheat, oilseed rape, sugar beet or maize might presumably increase the grain yield. Trees with reduced lignin contents or altered lignin structure will lead to a reduction in the cost of the paper as less lignin will have to be removed during the pulping process. On the other hand, novel papers may be produced due to the purity of cellulose fibre which could otherwise not be produced.

Reduction of lignification can be achieved by the application of chemical inhibitors to plants.

However, a more effective method controlling lignin deposition and structure is the inhibition of expression of the CAD gene using antisense RNA. Antisense RNA technology is an appropriate molecular biology approach to the inhibition of lignification. An antisense RNA is an RNA produced by the transcription of the non-coding DNA strand (nonsense). Thus, antisense RNA has the same sequence as the coding DNA strand and is complementary to the mRNA product of a specific gene.

As is well known, a cell manufactures protein by transcribing the DNA of the gene for that protein to produce RNA, which is then processed (e.g. by the removal of introns) into messenger RNA and finally translated by ribosomes into protein. This process may be inhibited by the presence in the cell of "antisense RNA". Therefore, as used herein, the term "antisense RNA" means an RNA sequence which is complementary to a sequence of bases in a mRNA: complementary in the sense that each base (or a majority of bases) in the antisense sequence (read in the 3′ to 5′ sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5′ to 3′ sense. It is believed that this inhibition takes place by formation of a complex between the two complementary strands of RNA, preventing the formation of protein. How this works is uncertain: the complex may interfere with further transcription, processing, transport or translation, or lead to degradation of the mRNA, or have more than one of these effects. Such antisense RNA may be produced in the cell by transformation with an

appropriate DNA construct arranged to transcribe backwards part of the coding strand (as opposed to the template strand) of the relevant gene (or of a DNA sequence showing substantial homology therewith).

The use of this technology to down-regulate the expression of specific plant genes has been described, for example in European Patent Publication No 271988 to ICI. Reduction of gene expression has led to a change in the phenotype of the plant: either at the level of gross visible phenotypic difference e.g. lack of lycopene synthesis in the fruit of tomato leading to the production of yellow rather than red fruit or at a more subtle biochemical level e.g. change in the amount of polygalacturonase and reduction in depolymerisation of pectins during tomato fruit ripening (Smith et al, Nature, 334, 724-726, 1988; Smith et al, Plant Mol Biol 14, 369-380, 1990). Thus antisense RNA has been proven to be useful in achieving down-regulation of gene expression in plants.

An object of the present invention is to provide plants having an altered ability to synthesise lignin.

According to the present invention there is provided the DNA insert contained in the clones pPLC4 and pTOMTI and variants thereof such as are permitted by the degeneracy of the genetic code or the functional equivalents thereof.

In addition, the present invention provides a recombinant DNA construct containing the said DNA under control of a transcriptional control sequence operative in plants, so that the construct can

generate mRNA in plant cells.

For the down-regulation of lignin synthesis the aforesaid DNA is in antisense orientation.

For the amplification of lignin biosynthesis the aforesaid DNA is in sense orientation thus to provide one or more additional copies of the said DNA in the plant genome.

Thus, in a further aspect, the present invention provides DNA constructs comprising a transcriptional initiation region operative in plants positioned for transcription of a DNA sequence encoding RNA complementary to a substantial run of bases showing substantial homology to an mRNA encoding the protein produced by the gene in pPLC4 pTOMT1.

The invention further provides plant cells, and plants derived therefrom having stably incorporated in their genomes the aforesaid DNA in sense or antisense orientation, and fruit and seeds of such plants. The present invention is principally concerned with the suppression of lignin formation and, that being so, the inserted gene will be in antisense orientation, but there are instances where over-production of lignin may have an advantageous effect, for example to improve plant stalk strength and resistance to diseases, and the present invention provides means for achieving amplification of the lignin biosynthetic ability of plants.

Thus the invention relates generally to the regulation of the plant's lignin biosynthetic pathway, in which OMT plays a dominant role, in order that the production of OMT, and hence the production and composition of lignin is altered by

insertion of the OMT gene, or a portion thereof (usually of 50 or more bases), in antisense orientation so that the amount of OMT for catalysing lignin synthesis is reduced.

The constructs of the invention may be inserted into plants to regulate the production of the CAD enzyme. Depending on the nature of the construct, the production of the protein may be increased, or reduced, either throughout or at particular stages in the life of the plant. It is also possible to target the expression of the gene to a specific cell types of the plant, such as the epidermis, the xylem, the roots etc.

The plants to which the present invention can be applied include commercially important food and forage plants, such as alfalfa, maize, oil seed rape, forage grasses and sunflower, and but also tree crops such as eucalyptus, pine species and poplar.

DNA constructs according to the invention preferably comprise a sequence of at least 50 bases which is homologous to the DNA of the insert in pPLC4 or pOMT1.A and pOMT1.B There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences between 100 and 1000 bases in length. The preparation of such constructs is described in more detail below.

The preferred source of antisense RNA for use in the present invention is DNA derived from the clone pPLC4 or pOMT1.A and pOMT1.B The required DNA encoding antisense RNA can be obtained in several ways: by cutting an appropriate sequence of

DNA from pPLC4 or pOMT1.A or pOMT1.B (or any other source of the OMT gene); by synthesising a DNA fragment using synthetic oligonucleotides which are annealed and then ligated together in such a way as to give suitable restriction sites at each end; by using synthetic oligonucleotides in a polymerase chain reaction (PCR) to generate the required fragment with suitable restriction sites at each end. The DNA is then cloned into a vector containing upstream promoter and downstream terminator sequences, the cloning being carried out so that the DNA sequence is inverted with respect to its orientation to the promoter in the strand from which it was cut. In the new vector, the strand that was formerly the template strand becomes the coding strand, and vice versa. The new vector will thus encode RNA in a base sequence which is complementary to the sequence of pPLC4 and pTOMT1 mRNAs. Thus the two RNA strands are complementary not only in their base sequence but also in their orientations (5' to 3').

As source of the DNA base sequence for transcription, it is convenient to use a cDNA clone such as pPLC4. The base sequence of pPLC4 is shown in Figure 1 and the base sequences of pOMT1.A and pOMT1.B is shown in Figure 2.

The clone pPLC4 has been deposited at the National Collections of Industrial and Marine Bacteria, PO Box 31, of 23 St Machar Drive, Aberdeen AB2 1RY, Scotland, as a plasmid in E.coli, strain 'sure', under the reference NCIB 40436 on August 22, 1991.

The clone pTOMT1.A has been deposited at the National Collections of Industrial and Marine

Bacteria, PO Box 31, of 23 St Machar Drive, Aberdeen AB2 1RY, Scotland, as a plasmid in E.coli, strain DH5α, under the reference NCIB 40439 on September 4, 1991.

The clone pTOMT1.B has been deposited at the National Collections of Industrial and Marine Bacteria, PO Box 31, of 23 St Machar Drive, Aberdeen AB2 1RY, Scotland, as a plasmid in E.coli, strain DH5α, under the reference NCIB 40440 on September 4, 1991.

A source of DNA for the base sequence for transcription is the promoter of the OMT gene itself or other genes involved in lignification such as the promoter of the phenyl alanine ammonia lyase gene or its modified version which permits expression in xylem tissue, or the s-Adenosyl methionine synthase gene or the promoter of the extensin gene. Such a gene may differ from the cDNA of pPLC4 and pOMT1A or pOMT1.B in that introns may be present. The introns are not transcribed into mRNA (or, if so transcribed, are subsequently cut out). When using such a gene as the source of the base sequence for transcription it is possible to use either intron or exon regions.

A further way of obtaining a suitable DNA base sequence for transcription is to synthesise it ab initio from the appropriate bases, for example using Figure 1 and Figure 2 as a guide. Recombinant DNA and vectors according to the present invention may be made as follows. A suitable vector containing the desired base sequence for transcription (for example pPLC4 and pOMT1.A and pOMT1.B) is treated with restriction enzymes to out cut the sequence. The DNA strand so obtained is

cloned (in reverse orientation) into a second vector containing the desired promoter sequence (for example cauliflower mosaic virus 35S RNA promoter or the bean PAL promoter, Bevan et al, EMBO J.8, 1899-1906 1988) and the desired terminator sequence (for example the 3' of the Agrobacterium tumefaciens nopaline synthase gene.

In this invention we may use both constitutive promoters (such as cauliflower mosaic virus 35S RNA) and inducible or developmentally regulated promoters (such as the PAL gene promoter) as circumstances require. Use of a constitutive promoter will tend to affect functions in all parts of the plant: while by using a tissue specific promoter, functions may be controlled more selectively. The use of a tissue-specific promoter, has the advantage that the antisense or sense RNA is only produced in the tissue in which its action is required.

Vectors according to the invention may be used to transform plants as desired, to make plants according to the invention. Dicotyledonous plants, such as alfalfa, oil seed rape etc, may be transformed by Agrobacterium Ti plasmid technology, for example as described by Bevan (1984) Nucleic Acid Research, 12, 8711-8721. Such transformed plants may be replicated sexually, or by cell or tissue culture.

Poplar and aspen transformation using Agrobacterium tumefaciens can be performed as described by De Block [Plant Physiol. (1990) 93:1110-1116]. Stem internode pieces are used as a tissue source for incubation with an Agrobacterium tumefaciens strain (C58CRif^R(pMP90.) harbouring

chimeric marker genes (bar/neo) on its non-oncogenic T-DNA. For the aspen clone (Populus alba x P. tremula; clone 357, Afocel) and the poplar clone (Populus trichocarpa x P. deltoides; clone 064, Afocel), transgenic shoots were obtained 3 months and 6 months after incubation, respectively.

The degree of production of RNA in the plant cells can be controlled by suitable choice of promoter sequences, or by selecting the number of copies, or the site of integration, of the DNA sequences according to the invention that are introduced into the plant genome. In this way it may be possible to modify lignification to a greater or lesser extent.

The constructs of our invention may be used to transform cells of both monocotyledonous and dicotyledonous plants in various ways known to the art. In many cases such plant cells (particularly when they are cells of dicotyledonous plants) may be cultured to regenerate whole plants which subsequently reproduce to give successive generations of genetically modified plants. Examples of genetically modified plants according to the present invention include, alfalfa, oil seed rape, sunflower, sorghum, maize, festuca, and trees such as eucalyptus, poplar, and pine.

In the present invention, we use antisense RNA in order to determine the phenotype of transgenic plants which show modified, that is increased or reduced, expression of pPLC4 or pTOMT1 by the use of antisense and sense expression vectors.

The invention will now be described further with reference to the accompanying drawings, in which:

Figure 1 shows the complete nucleotide sequence and deduced amino acid sequence from pPLC4.

Figure 2 shows the combined nucleotide sequence and deduced amino acid sequence of pOMT1.A and pOMT1.B.

Figure 3 shows the nucleotide sequence of a complete cDNA clone of OMTI from a stem tobacco library.

Figure 4 is the nucleotide sequence of a OMT III cDNA isolated from a λZapII library of tobacco leaf.

Figure 5 shows the construction of antisense and sense vectors using the 5' end 500 bp and the 3' end 900 bp BamH1 fragments from pPLC4.

Figure 6 shows the construction of an antisense vector using a 1.4 kb PCR fragment containing the complete pPLC4 clone.

The invention will now be described by way of illustration in the following Examples.

EXAMPLE 1

Purification of poplar OMTs

A. Isolation procedure

Table 1 shows the OMT activity using caffeic acid as a substrate in young leaves and xylem tissue from poplar trees (Populus trichocarpa x P. deltoides).

TABLE 1

OMT Activity in young leaves and xylem tissue from poplar trees

Tissue	Activity/g tissue n kat/g	Specific Activity n kat/g protein
Leaves	0.015	0.002
Xylem	0.6	0.2

There is approximately 50-fold more caffeic acid O- methyltransferase activity in xylem as compared to leaves. Subsequently, OMT activity was purified both from leaves and xylem tissue. The procedure for the purification of the OMTs was established by Dumas et al., (1988). The purification of OMT activity from a total protein extract of poplar leaves via ammonium sulphate precipitation, desalting on Sephadex G25, Q-Sepharose chromatography, and adenosine agarose affinity chromatography, and finally, MonoQ chromatography resulted in one 38-kDa protein. About 5 μg of OMT was obtained from 100 g of leaves. Using xylem as source, several purification steps were omitted. Crude protein extract from 5 g of xylem was applied immediately on an agarose adenosine column leading to the purification of about 50 μg of the 38- kDa OMT with a yield of 50% . At this stage a minor contaminating band of 37-kDa is still visible.

B. Immunological characterisation of poplar OMTs.

Antibodies raised against OMT I and OMT II from tobacco were used to test for cross-reactions

with the poplar OMTs. Antibodies raised against OMT II cross-react with the proteins (Mr 38 kDa and 70 kDa) on protein gel blots of proteins purified from leaves by three purification steps (G25, Q-Sepharose, and adenosine agarose affinity chromatography). Antibodies raised against OMT I show weak cross- reaction with a 37-kDa protein. A fourth purification step (chromatography on MonoQ column) resulted in one 38-kDa protein that cross-reacts solely with antibodies directed against OMT II on protein gel blots.

C. Inhibition in vitro of the poplar OMT caffeic acid O-methyltranferase activity by antibodies against OMT II and OMT I from tobacco.

Although the poplar OMT and the tobacco OMT II have a different substrate specificity, the protein gel blots clearly indicate that both enzymes must have similar epitopes recognised by the rabbit antiserum against tobacco OMT II.

Five ng of purified poplar OMTI was mixed with corresponding amounts of antibodies directed against tobacco OMTI and OMTII. After incubation at 37°C, OMT activity towards caffeic acid was measured. The results are given in Table 2 which shows that the caffeic acid OMT activity of the purified poplar OMT can be inhibited by adding antibodies directed against OMT II from tobacco prior to the OMT activity assay (see Materials and Methods). Two and four µl of undiluted rabbit antiserum against tobacco OMT II mixed with purified poplar OMT resulted in a 87% and 92% inhibition, respectively, of the OMT activity. However, 2 µl of undiluted anti-OMT I rabbit antiserum mixed with purified poplar OMT led to a

29% inhibition of the OMT activity. No inhibition of the OMT activity was observed by mixing 2 µl of pre-immune serum with the purified poplar OMT prior to the OMT activity assay.

TABLE 2

Inhibition in vitro of the poplar OMT activity by antibodies directed against OMTI and OMTII from tobacco

Antibody	OMT activity toward Caffeic acid (%)	Inhibition (%)
-	100	0
2µl anti-OMTI	71	29
2µl anti-OMTII	13	87
4µl anti-OMTII	8	92
2µl pre-immune serum	100	0

D. Specific activity of the purified poplar OMT towards different phenolic compounds.

The specific activity of the purified OMT towards three different O-diphenolic substrates was measured. Using catechol, caffeic acid, and hydroxyferulic acid as substrate, we found an OMT activity of 0, 30, and 15 nkat/mg protein, respectively.

Previously, it has been shown that OMT 1 from tobacco uses mainly caffeic acid and hydroxyferulic acid as a substrate (Collendavelloo et al., 1981). Therefore, the purified poplar OMT has an OMT I-like enzymatic activity.

EXAMPLE 2

Microsequencing of the 38-kDa OMT.

The 38-kDa protein isolated from poplar xylem tissue was digested with trypsin and the peptides were separated on reverse-phase HPLC. Four peptides were sequenced:

peptide 45 (R/KDLPHVIEDAPSYGVEHVGGDMF)

peptide 49 (LVDVGGGTGAVV)

peptide 51 (GINFDLPHVIEDAP)

peptide 52 (VILVE?ILPVAPD).

Note that peptide 45 is a mixture of two peptides with arginine and lysine, respectively, as first amino acid. Trypsin cleaves proteins after a lysine or arginine residue, except when this is followed by proline, glutamic acid, or aspartic acid. This implies that the first amino acid preceding the sequence of peptide 45 has to be either an arginine or a lysine. The sequences of peptide 45 and 51 are overlapping.

EXAMPLE 3

Design of oligos and cloning of OMT sequences.

Two amino acid sequences of peptide 49 (VDVGGGTGA) and peptide 51 (PHVIEDAP) were chosen to design degenerated oligonucleotides which were subsequently used as primers for a PCR with DNA prepared from a leaf cDNA library of poplar. Since the relative position of the peptide in the protein are unknown, both sense and antisense oligonucleotides were designed. Following the PCR, a 108-bp fragment was found to be amplified with sense primer 49 and antisense primer 51. Using both total RNA from xylem and genomic DNA of poplar, as a template, the same fragment was amplified. The PCR product was subcloned as a

blunt-end fragment in the SmaI site of pGem2 yielding plasmid pPLC1. The nucleotide and deduced amino acid sequences are shown in Figure 1 (positions 661-768).

To isolate a full-length cDNA, the 108-bp fragment was used as a probe to screen a leaf cDNA library from poplar. Three different clones, designated pPLC2, pPLC3, and pPLC4 were identified out of 80,000 clones. The nucleotide sequence of pPLC4 and the deduced amino acid sequence are shown in Figure 1. The cDNA insert is 1375 nucleotides in length and contains one open reading-frame of 1092 nucleotides encoding a protein of 364 amino acids (calculated M 39,720; Pi 5.45). The pPCL2 clone contains a cDNA of 1,420 nucleotides and contains also one ORF of 1,092 nucleotides encoding a protein of 364 amino acids. There is only a 3-amino acid difference between the proteins of the pPCL4 and the pPCL2 clones. Amino acids 97, 191, and 361 in the protein of pPCL2 are leucine, isoleucine, and phenylalanine, respectively. Thus a mixture of at least three closely related isoforms, as shown by the two isoforms present in peptide 45 and by the amino acid sequence of peptide 51 which is entirely found back in the deduced OMT sequence.

EXAMPLE 4

<u>Analysis of the transgenic poplar trees for a modified lignin content</u>

<u>A. Transformation of poplar</u>

With the poplar OMT cDNA clone (pPCL4) five different sense and antisense constructs were made resulting in five plasmids: p35SSOM3A (a 500 bp 5'end fragment in sense), p35SASOM3A (a 500 bp 5' end fragment in antisense), p35SSOM3B (a 900 pb 3'

end fragment in sense), p35SASOM3B (a 900 pb 3' end fragment in antisense) and p35SASOM3C (the full length OMT clone in antisense). All the constructs are under the control of the constitutive 35S Cauliflower Mosaic Virus (CaMV) promoter. These constructs were introduced in poplar via an 'Agrobacterium tumefaciens' - mediated transformation of stem explants.

For the plasmids p35SSOM3A (SA), p35SASOM3A (ASA), p35SSOM3B (SB) and p35SASOM3B (ASB) 16,21,7 and 13 independent transformants were regenerated, respectively. Control transformations were performed using plasmid pGSJ780A (this plasmid contains only the 35S CaMV promoter).

2. Estimation of the OMT antisense RNA amount in leaves from ASA and ASB plants.

Using single stranded (ribo) probes (which only hybridise with the antisense RNA) RNA gel blots were performed. The antisense B RNA was detected (strongly) in candidate ASB 5B and candidates ASB 3Am ASB 5A and ASB 7A.

The antisense A RNA levels were high in candidates ASA 1A and candidates ASA 5B, ASA 17A, ASA 6A and ASA 2B. No antisense RNA was detected in transgenic plants containing the pGSJ780A T-DNA. The difference in the antisense RNA levels can be explained by position effects.

3. Steady-state OMT sense RNA in leaves from SA and SB plants.

In an analogous way, by performing Northern blots with single stranded riboprobes which only detect sense RNA, SA and SB plants were analysed.

The sense B RNA can be detected easily in candidates SB 5A and SB 8A and not in candidates SB

2B, SB 4A, SB 7A and SB 10A.

The sense A RNA amount is high in candidates SA 11A and SA 12A, low in candidates SA 9A, SA 18A and SA 23A.

In all the transformants the endogenous OMT mRNA could not be detected (this is in agreement with the fact that the OMT is strongly expressed in xylem and very weak in leaves). All these different transgenics have the same phenotype as the wild type poplar and control plants.

4. OMT activity towards caffeic acid in the transgenic plants.

The OMT activity was measured in different organelles/tissues (Tables 3 and 4). Two candidates (p35SASOM3B 4A and 6A) with a lower OMT activity in petioles, xylem and phloem (two to three times lower) in comparison to wild type and control plants, were identified.

For both these candidates, there was no correlation between the reduced OMT activity and the amount of antisense RNA present in these plants.

5. Lignin analysis of the transgenic poplars.

The transgenic plants were analysed for their lignin composition (Table 5). Lignin is a complex polymer of three different units: p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomers. Poplar lignin contains both guaiacyl and syringyl units. A typical syringyl/guaiacyl (S/G) ratio for poplar is 2/1. Due to the antisense inhibition we expect that the monomer ratio will be modified, resulting in a lower S/G ratio. Lignin characterisation of xylem was performed by the analysis of degradation products recovered from

thioacidolysis. This method allows the estimation of the different units, involved in the lignin characteristic structure.

For the wild type and control plants a typical S/G ratio was found (2/1, Table 5). However, the two candidates ASB 4A and ASB 6A have a lower S/G ratio, 1.47 and 1.43, respectively. Like the bm3 maize mutants (these mutants have a lower S/G ratio (.039) in comparison with wild type maize (1.72) we notice that the S/G ratio of the two ASB poplars has been modified (although not as drastically as was the case for the maize mutants).

TABLE 3
OMT activity in petioles of the different ASB, ASA, SB and SA plants (in cpm).

Sample		Replicates 1	2	3	Mean
ASB	6A	-	2460	1252	1856
	8A	6074	5710	6931	6238
	4A	1097	1946	711	1251
	5B	2419	6295	5764	4826
ASA	23A	1954	4342	3834	3376
	17A	5001	7596	5386	5994
	2B	2602	3926	5796	4108
SB	4A	2527	5799	5226	4517
	10A	2252	4970	5631	4284
	5A	4726	4672	4838	4755
	7A	2686	6302	7490	5492
SA	13A	3638	5423	3697	4252
	3B	3269	4201	6875	4781
	2B	2479	6626	5842	4982
	7A	5338	8244	8691	7424
WT	T1	3231	2843	7577	4550
	T2	3996	6171	6433	5533
	T3	3470	5152	10028	6216

WT = Control Plants.

TABLE 4

OMT activity in xylem and phloem of the candidates ASB 4A and ASB 6A (in cpm). Mean value, last lane.

Sample		Replicates 1	2	3	Mean
ASB	4AX	2465	1624	743	1610
	F	3530	1108	1482	2040
ASB	6AX	3089	1845	1152	2028
	F	4258	2063	3143	3154
WT	T1X	5040	4440	2262	3914
	F	8665	5505	8809	7659

WT = Control plants

TABLE 5

Syringyl/guaiacyl ratio of the different ASB, ASA, SB and SA plants.

Sample		Replicates 1	2	Mean
ASB	18A	2.17	2.41	2.29
	5A	1.61	1.84	1.73
	4A	1.34	1.60	1.47
	6A	1.28	1.59	1.43
ASA	7B	1.93	2.48	2.20
	15A	1.80	2.35	2.07
	23A	1.88	2.13	2.00
SB	4A	1.95	2.09	2.02
SA	1B	2.07	2.20	2.14
WT	T1	2.02	1.98	2.00

WT = Control Plants

EXAMPLE 5

Isolation of a tobacco OMT1 clones pTOMT1A and pTOMT1B.

Using similar procedures described for the isolation of the poplar OMT cDNA, a tobacco OMT1 cDNA clones were isolated from a leaf cDNA library prepared from RNA of TMV infected tobacco leaves. These clones were isolated by PCR using sequence information of the purified tobacco OMT1 protein. The combined sequence of two clones covering the complete coding sequence of tobacco OMT1 are shown in Figure 2. The underlined part represent sequences found in clone A. the dotted part represents sequences found in clone B.

A complete cDNA clone of OMTI has been isolated from a stem cDNA tobacco library. Its sequence is shown in Figure 3.

EXAMPLE 6

Immunoscreening of a λgt11 expression library and characterisation of the MOT III cDNA clone o3.614

The λgt11 phages were plated on Escherichia coli Y1090 and fusion proteins induced with 10mM IPTG. The proteins bound to nitro-cellulose filters were screened by immunodetection using anti-tobacco OMT III polyclonal antibody (Hermann et al., 1987; Dumas, 1990). Three positive clones were purified after immunoscreening of the λgt11 library. The length of their cDNA inserts was determined by PCR. Aliquots of phage lysates (5μl) were heated for 5 mins at 94°C and used as template for PCR. The amplification mixture consists of 10mM Tri-HCl pH 8.3, 11mM KCl, 1.6mM $MgCl_2$, 1mM DTT, 200μM each dNTP, 1μM each oligonucleotide primer, and 1 unit of Taq polymerase (Beckman) in a

final volume of 50µl. The amplification program consisted of 25 cycles of denaturation (94°C, 1 min), annealing (40°C, 1 min), and primer extension (72°C, 1 min). The longest clone, named o3.614, was 614 bp long and was submitted to phage amplification and DNA purification by CsCl gradient (4). The 614 bp cDNA insert was subloned by EcoRI restriction digestion in pBluescript KS(+)(Stratagene, Inc). According to standard methods (4), deletion from both the extremities of the plasmid were generated by ExoIII-Mung Bean digestions and the primers and the T7 DNA polymerase.

EXAMPLE 7

Construction and screening of a λZapI-I cDNA library from tobacco leaf RNA and characterisation of a complete clone

Poly(A)+RNA from 48 hours TMV infected leaves was used to construct an oligo(dT) primed cDNA. Double-stranded cDNA was ligated to hemi-phosphorylated EcoR I/Not I adaptors (Pharmacia), ligated into λZapII vector (Stratagene, Inc) and packaged using Gigapack in vitro packaging extracts (Stratagene, Inc). The resulting cDNA library titled 1.8×10^7 pfu/µg cDNA. The o3.614 clone was used as DNA probe. The 614 bp DNA fragment was purified from purified from 0.8% agarose gel (PrepAGene, BIORAD), 32p labelled by random oligonucleotide-primed synthesis and used to screen a λZapII library made by standard protocols (4). After three cycles of screening, twelve positive clones were isolated from approximately 1.8×10^5 plaque-forming units. cDNA inserts from positive phage were rescued as

Bluescript plasmids by R408 helper phage mediated in vivo excision, as described by the manufacture (Stratagene, Inc). Cloned insert DNA was isolated by Not I digestion and analysed on 1% agarose gels. The cDNA clones isolated from λZapII cDNA library were mapped with restriction endonucleases and then sequenced on both strands by the dideoxy chain termination method 95) using T7 DNA polymerase (Pharmacia). For sequencing the internal regions, synthetic oligonucleotide primers (18mers) were designed from the DNA sequence previously determined. The complete sequence of a OMT III cDNA clone is given in Figure 4. The amino acid sequences of peptides obtained by trypsic digestion of the purified OMT II and OMT III proteins all correspond to the aminoacid sequence derived from OMT III cDNA clones. These evidences suggest the occurrence of only one mRNA species for the "Pathogenesis-Related" forms OMT II and OMT III but of 2 forms for the lignification-related OMT I.

EXAMPLE 8

Localisation of OMT I transcripts by in situ hybridisation.

In petiole sections, OMT I mRNAs were localised in parenchyma cells of xylem and phloem, with a marked signal around the nuclei. In leaf sections, OMT I mRNAs were found to accumulate particularly in the upper and lower epidermis in a ring of tissue surrounding TMV-induced necrotic lesions, were no cell-type specific hybridisation was found in the healthy leaf.

EXAMPLE 9

Design of antisense vectors

Five different poplar OMT antisense constructs

have been made. A 500 bp BamHI fragment corresponding to the 5' end of the poplar OMT clone and a 900-bp BamHI fragment corresponding to the 3' end of the poplar OMT clone were cloned in the BamHI site of vector pGSJ780A, in both sense and antisense direction yielding the four plasmids p35SSOM3A, p35SASOM3A, p35SASOM3A, p35SSOM3B, and p35SASOM3B (Figure 5). All OMT fragments are under the control of the CaMV 35S promoter. The full-length OMT clone was cloned in the vector pGSJ780A in antisense direction by PCR yielding plasmid p35SASOM3C (Figure 6). The correct direction of the inserts has been confirmed by sequencing. The vector pGSJ780A is a binary vector with the pVS1 origin of replication and a Sm/Sp resistance gene for selection in Agrobacterium tumefaciens. Between the T-DNA borders there is a nos-nptII-ocs cassette and a multiple cloning site).

The construction of the tobacco antisense vector follows that described for the poplar vectors. The insert was inserted into the vector pGSJ780A.

CLAIMS

1. A DNA encoding caffeic acid O-methyl transferase contained in the clones pPLC4 and pTOMTI and variants thereof such as are permitted by the degeneracy of the genetic code or the functional equivalents thereof.

2. A recombinant DNA comprising the DNA claimed in claim 1 under control of a transcriptional control sequence operative in plants.

3. A recombinant DNA as claimed in claim 2, for the down-regulation of lignin biosynthesis in which the said DNA is in antisense orientation.

4. A recombinant DNA as claimed in claim 2, for the amplification of lignin biosynthesis, in which the said DNA is in sense orientation.

5. A DNA comprising a transcriptional initiation region operative in plants operatively linked to a DNA sequence encoding RNA complementary to a substantial number of bases showing substantial homology to an mRNA encoding the protein produced by the gene in pPLC4 or pTOMT1 so as to initiate production of mRNA therefrom.

6. A DNA having the nucleotide sequence given in Figure 1.

7. A DNA having the nucleotide sequence given in Figure 2.

8. A DNA having the nucleotide sequence given in Figure 3.

9. A DNA having the nucleotide sequence given in Figure 4.

10. A plant cell, and a plant derived therefrom having stably incorporated in its genome by transformation a DNA as claimed in any one of claims 1 to 9 in sense or antisense orientation, and the fruit and seeds of said plants.

11. A method for the regulation of lignin biosynthesis in a plant comprising stably incorporating into the genome of a plant by transformation a recombinant DNA encoding the enzyme caffeic acid O-methyl-transferase in sense or antisense orientation under control of a transcriptional initiation region operative in plants.

FIG. 1

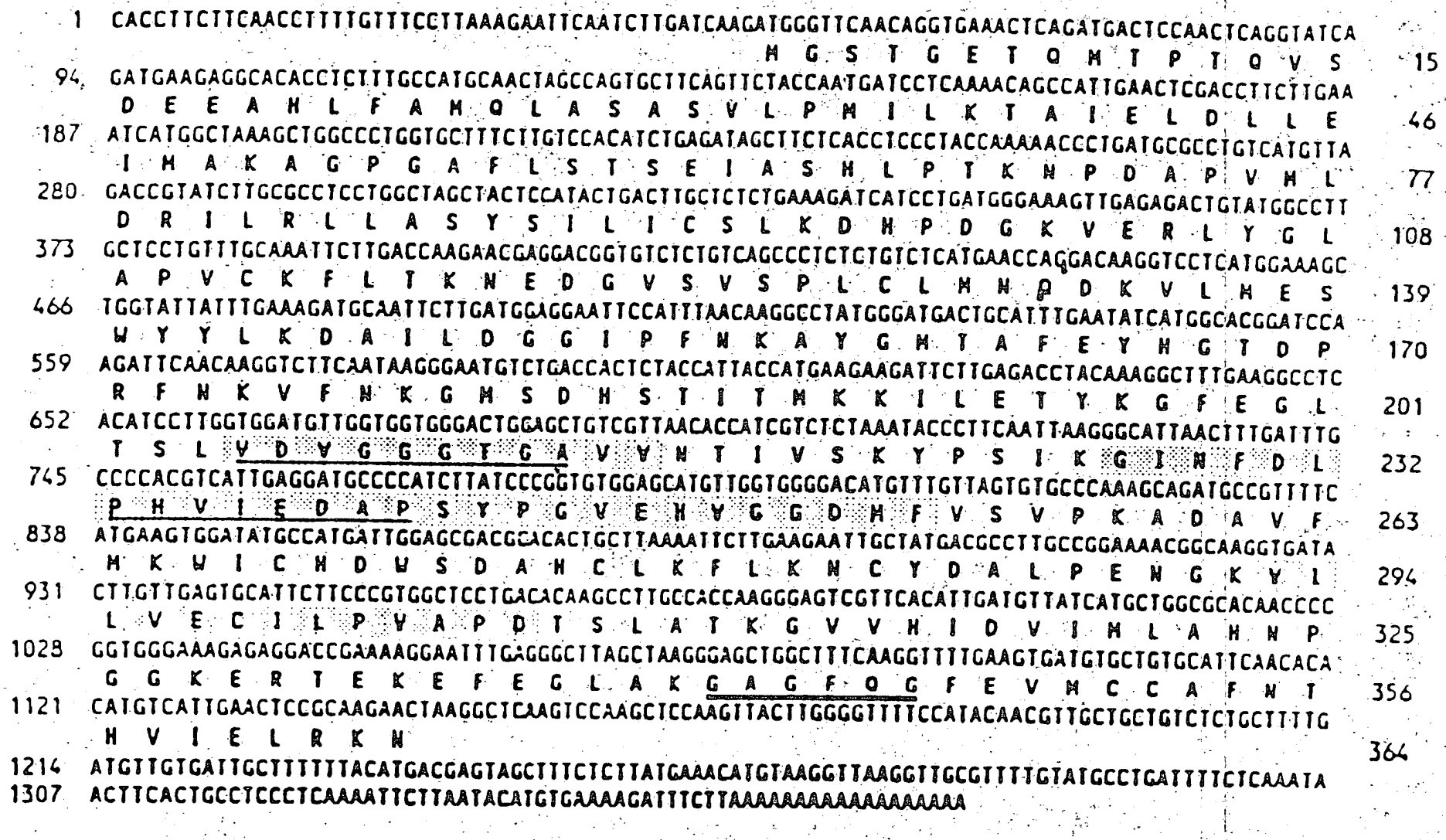
1 CACCTTCTTCAACCTTTTGTTTCCTTAAAGAATTCAATCTTGATCAAGATGGGTTCAACAGGTGAAACTCAGATGACTCCAACTCAGGTATCA
M G S T G E T Q M T P T Q V S 15
94 GATGAAGAGGCACACCTCTTTGCCATGCAACTAGCCAGTGCTTCAGTTCTACCAATGATCCTCAAAACAGCCATTGAACTCGACCTTCTTGAA
D E E A H L F A M Q L A S A S V L P M I L K T A I E L D L L E 46
187 ATCATGGCTAAAGCTGGCCCTGGTGCTTTCTTGTCCACATCTGAGATAGCTTCTCACCTCCCTACCAAAAACCCTGATGCGCCTGTCATGTTA
I M A K A G P G A F L S T S E I A S H L P T K N P D A P V M L 77
280 GACCGTATCTTGCGCCTCCTGGCTAGCTACTCCATACTGACTTGCTCTCTGAAAGATCATCCTGATGGGAAAGTTGAGAGACTGTATGGCCTT
D R I L R L L A S Y S I L I C S L K D H P D G K V E R L Y G L 108
373 GCTCCTGTTTGCAAATTCTTGACCAAGAACGAGGACGGTGTCTCTGTCAGCCCTCTCTGTCTCATGAACCAGGACAAGGTCCTCATGGAAAGC
A P V C K F L T K N E D G V S V S P L C L M N Q D K V L M E S 139
466 TGGTATTATTTGAAAGATGCAATTCTTGATGGAGGAATTCCATTTAACAAGGCCTATGGGATGACTGCATTTGAATATCATGGCACGGATCCA
W Y Y L K D A I L D G G I P F N K A Y G M T A F E Y H G T D P 170
559 AGATTCAACAAGGTCTTCAATAAGGGAATGTCTGACCACTCTACCATTACCATGAAGAAGATTCTTGAGACCTACAAAGGCTTTGAAGGCCTC
R F N K V F N K G M S D H S I I T M K K I L E T Y K G F E G L 201
652 ACATCCTTGGTGGATGTTGGTGGTGGGACTGGAGCTGTCGTTAACACCATCGTCTCTAAATACCCTTCAATTAAGGGCATTAACTTTGATTTG
T S L V D V G G G T G A V V N T I V S K Y P S I K G I N F D L 232
745 CCCCACGTCATTGAGGATGCCCCATCTTATCCCGGTGTGGAGCATGTTGGTGGGGACATGTTTGTTAGTGTGCCCAAAGCAGATGCCGTTTTC
P H V I E D A P S Y P G V E H V G G D M F V S V P K A D A V F 263
838 ATGAAGTGGATATGCCATGATTGGAGCGACGCACACTGCTTAAAATTCTTGAAGAATTGCTATGACGCCTTGCCGGAAAACGGCAAGGTGATA
M K W I C H D W S D A H C L K F L K N C Y D A L P E N G K V I 294
931 CTTGTTGAGTGCATTCTTCCCGTGGCTCCTGACACAAGCCTTGCCACCAAGGGAGTCGTTCACATTGATGTTATCATGCTGGCGCACAACCCC
L V E C I L P V A P D T S L A T K G V V H I D V I M L A H N P 325
1028 GGTGGGAAAGAGAGGACCGAAAAGGAATTTGAGGGCTTAGCTAAGGGAGCTGGCTTTCAAGGTTTTGAAGTGATGTGCTGTGCATTCAACACA
G G K E R T E K E F E G L A K G A G F Q G F E V M C C A F N T 356
1121 CATGTCATTGAACTCCGCAAGAACTAAGGCTCAAGTCCAAGCTCCAAGTTACTTGGGGTTTTCCATACAACGTTGCTGCTGTCTCTGCTTTG
H V I E L R K N 364
1214 ATGTTGTGATTGCTTTTTTACATGACGAGTAGCTTTCTCTTATGAAACATGTAAGGTTAAGGTTGCGTTTTGTATGCCTGATTTTCTCAAATA
1307 ACTTCACTGCCTCCCTCAAAATTCTTAATACATGTGAAAAGATTTCTTAAAAAAAAAAAAAAAAAA

FIG. 2 (1/2)

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   1  GCCGGCCCGG GCGCGGCGAT TTCTCCTTCT GAATTAGCTG CTCAGCTCTC
  51  AACCCAGAAC CCAGAAGCAC CCGTTATGCT TGATCGGATG CTTAGGCTAC
 101  TTGCTACTTA CTCTGTTCTC AATTGTACTC TTAGAACACT GTCTGATGGC
 151  AGTGTTGAGA GGCTTTATAG TCTGGCTCCG GTTTGTAAGT TCTTGACTAA
 201  GAATGCTGAT GGTGTTTCTG TTGCCCCACT TTTGCTTATG AATCAAGATA
 251  AAGTTCTTAT GGAGAGCTGG TACCACTTAA AAGATGCAGT ACTAGATGGT
 301  GGAATCCCAT TCAACAAGGC CTATGGAATG ACAGCATTTG AGTACCATGG
 351  CACAGATCCA AGATTCAACA AAGTTTTCAA CCGTGGAATG TCTGATCACT
 401  CCACTATGTC AATGAAAAAG ATTCTTGAGG ACTACAAAGG ATTTGAAGGC
 451  CTAAATTCCA TTGTCGATGT TGGTGGTGGA ACTGGCGCTA CTGTTAACAT
 501  GATTGTCTCC AAACATCCCT CTATTAAGGG TATTAACTTT GATTTACCAC
 551  ATGTTATTGG AGATGCTCCA GCTTACCCTG GTGTCGAGCA CGTTGGTGGC
 601  GACATGTTTG CCAGTGTGCC AAAAGCAGAT GCCATTTTCA TGAAGTGGAT
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651 TTGTCATGAT TGGAGCGACG AGCATTGCCT AAAATTCTTG AAGAATTGCT

701 ATGAAGCACT ACCTGCAAAT GGGAAGGTGA TAATAGCGGA GTGCATACTT

751 CCAGAGGCCC CAGATACATC ACTTGCAACT AAGAATACAG TACATGTTGA

801 TATTGTGATG TTAGCACATA ACCCAGGAGG CAAAGAAAGG ACTGAGAAGG

851 AATTTGAGGC TTTGGCTAAG GGCGCTGGTT TTACTGGATT CGCAAGGCTT

901 GTTGCGCTTA CAACACTTGG GTCATGGAAT TCAACAAATA ATTAATCGAT

951 TCCTTTGGAG AATTAAGCAA TATACTGTTC ATTTTGCATT TTGAAATTCT

1001 ACTTTTCACA GAGTGGCTTT ACTGCGAAAT AAAAGAAATA TATAGCTTTT

1051 ACCTTGAAAA GATCAATGTT CAAAGGGAAA AAAAAAAGGA AGATGAAATA

1101 ATTGCTCTCA GAAAAGCAGT GTGTTAGGAA AAAGCTTTTT AGCTGGATTT

1151 TGAATTTTTA TTGTATGTAT TTCTGTAATA CACATGTATT GAAGGAATAC

1201 TAGTTTTCGA CCAATCATAT TTCTTTGAAA AAAAAAAAAA AAAA

FIG.2(2/2)

FIG. 3 (1/2)

```
ATTCCTTCAACTTACCCAATTAAGTCATCGAAAAATCTGAAACAGAACTAAAAGTAAAAT
1
                                                              M
TTATTTGCCATGCAATTGTGTAGTGCTTCTGTACTTCCTATGGTCCTAAAATCAGCCGTAGA
119
 L  F  A  M  Q  L  C  S  A  S  V  L  P  M  V  L  K  S  A  V  E
21
GAATTAGCTGCTCAGCTCTCAACTCAGAACCCAGAAGCACCTGTTATGCTTGATCGGATGCT
239
 E  L  A  A  Q  L  S  T  Q  N  P  E  A  P  V  M  L  D  R  M  L
61
AGTGTTGAGAGGCTTTATAGTCTGGCTCCCGTCTGTAAGTACTTGACTAAGAATGCTGATGG
359
 S  V  E  R  L  Y  S  L  A  P  V  C  K  Y  L  T  K  N  A  D  G
101
TACCACTTAAAAGATGCAGTACTAGATGGCGGAATCCCATTCAACAAAGCCTATGGAATGCA
419
 Y  H  L  K  D  A  V  L  D  G  G  I  P  F  N  K  A  Y  G  M  T
141
TCTGATCACTCCACTATGTCAATGAAGAAGATTCTTGAGGACTACAAAGGATTTGAAGGCCT
599
 S  D  H  S  T  M  S  M  K  K  I  L  E  D  Y  K  G  F  E  G  L
181
AAATATCCCTCTATTAAGGGCATTAACTTTGATTTGCCACATGTAATTGGAGATGCTCCAAC
719
 K  Y  P  S  I  K  G  I  N  F  D  L  P  H  V  I  G  D  A  P  T
221
GCCATTTTCATGAAGTGGATTTGTCATGATTGGAGCGATGAGCATTGCCTAAAATTCTTGAA
839
 A  I  F  M  K  W  I  C  H  D  W  S  D  E  H  C  L  K  F  L  K
261
CCAGAGGCCCCAGATACATCACTTGCAACTAAGAATACAGTACATGTTGATATTGTTATGTT
959
 P  E  A  P  D  T  S  L  A  T  K  N  T  V  H  V  D  I  V  M  L
301
GGCGCTGGTTTTACTGGATTCGCAAGGCTTGTTGCGCTTACAACACTTGGGTCATGGAATTC
179
 G  A  G  F  T  G  F  A  R  L  V  A  L  T  T  L  G  S  W  N  S
341
CATTTGGAAATTCTACTTTTCTCAGAGTGGCTTGACTGTGAAATAAAAGAAATATAGCTTTT
1199
AAAGCAATGTGTTAGGAAAAAGCTTTTTTAGCTGGATTTTGAATTTTACTGTATGTATTTCT
1319
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FIG. 3 (2/2)

GGGTTCAACAAGCGAGAGCCAGAGTAACAGTCTCACTCACACAGAAGACGAAGCTTTC

G S T S E S Q S N S L T H T E D E A F 118

ACTTGACCTTCTTGAGCTAATGGCTAAGGCTGGTCCAGGTGCAGCTATTTCTCCTTCT 20

L D L L E L M A K A G P G A A I S P S 238

TAGGCTACTTGCTTCTTACTCTGTTCTCAATTGTACTCTTAGAACACTGCCTGATAGC 60

R L L A S Y S V L N C T L R T L P D S 358

TGTTTCTGTTGCCCCACTTTTGCTTATGAATCAAGATAAAGTTCTTATGGAGAGCTGG 100

V S V A P L L L M N Q D K V L M E S W 418

AGCATTTGAGTACCATGGCACAGATCCAAGATTCAACAAAGTGTTCAACCGTGGAATG 140

A F E Y H G T D P R F N K V F N R G M 598

AAATTCCATTGTTGATGTTGGTGGTGGAACGGGTGCTACTGTTAACATGATTGTCTCT 180

N S I V D V G G G T G A T V N M I V S 718

TTACCCCGGTGTCGAGCACGTTGGTGGCGACATGTTTGCTAGTGTGCCAAAAGCAGAT 220

Y P G V E H V G G D M F A S V P K A D 838

GAATTGCTATGAAGCACTACCTGCAAATGGGAAGGTGATAATTGCAGAGTGCATACTT 260

N C Y E A L P A N G K V I I A E C I L 958

AGCACATAACCCAGGAGGCAAAGAAAGGACTGAGAAGGAATTTGAGGCTTTGGCTAAG 300

A H N P G G K E R T E K E F E A L A K 1078

AACAAGTAATTAATCGATTCCTTAATTTGAAGGATTAAGCAATATACTGTTCGTTTTG 340

T S N * 1198

AACTTGAAAAGATTGATGTTCAAAAGAAAAAAAGGAAGATGAAATAATTGCTCTCAGA 364

GTTATACACATGTATTGAAGGAATACTAGTTTTCGACCAAAAAAAAAAAAA 1318

1431

FIG. 4 (1/5)

```
   1 AACAAAAACACTCTAAAAGGAAAAGACTAGGAGAATAATTACACTACAACTCTTATAGCT

  61 AATTCTTGTCTCAAGATTTTCACCTATGGAATCCTCAACCAAAAGCCAAATACCAACACA

                              M  E  S  S  T  K  S  Q  I  P  T  Q  12

 121 ATCAGAAGAAGAGCGTAACTGCACATATGCCATGCAACTATTGTCATCTTCAGTCCTCCC

      S  E  E  E  R  N  C  T  Y  A  M  Q  L  L  S  S  S  V  L  P  32

 181 CTTTGTGTTGCATTCAACAATTCAATTGGAAGTTTTTGAGATATTAGCCAAATCTAATGA

      F  V  L  H  S  T  I  Q  L  E  V  F  E  I  L  A  K  S  N  D  52

 241 CACTAAACTTTCTGCTTCTCAAATTGTTTCTCAAATTCCTAACTGCAAGAATCCTGATGC

      T  K  L  S  A  S  Q  I  V  S  Q  I  P  N  C  K  N  P  D  A  72
```

FIG. 4 (2/5)

```
301 AGCTACTATGTTAGATAGGATGCTTTATCTGTTGGCTAGTTACTCGTTGTTTACTTGTTC

    A  T  M  L  D  R  M  L  Y  V  L  A  S  Y  S  L  F  T  C  S  92

361 CATTGTTGAGGATGAAGAAAATAATGGGGGCCAGAAAAGAGTCTATGGTTTGTCACAAGT

    I  V  E  D  E  E  N  N  G  G  Q  K  R  V  Y  G  L  S  Q  V  112

421 GGGAAAATTCTTTGTTAGAGATGAAGATGGTGCATCAATGGGGCCACTTTTGGCTTTGCT

    G  K  F  F  V  R  D  E  D  G  A  S  M  G  P  L  L  A  L  L  132

481 TCAAGATAAAGTATTCATAAACAGCTGGTTTGAACTAAAAGATGCAGTTCTTGAAGGAGG

    Q  D  K  V  F  I  N  S  W  F  E  L  K  D  A  V  L  E  G  G  152

541 AGTTCCATTTGACAGGGTACACGGTGTTGTCCATGCATTTGAATATCCAAAATCGGACCC

    V  P  F  D  R  V  H  G  V  V  H  A  F  E  Y  P  K  S  D  P  172
```

FIG. 4 (3/5)

```
601 AAAATTCAATGATGTTTTCAACAAGGCAATGATCAATCACACAACTGTAGTCATGAAAAA

    K  F  N  D  V  F  N  K  A  M  I  N  H  T  T  V  V  M  K  K  192

661 AATACTTGAAAATTACAAAGGTTTTGAGAACCTTAAAACTTTGGTTGATGTTGGAGGTGG

    I  L  E  N  Y  K  G  F  E  N  L  K  T  L  V  D  V  G  G  G  212

721 TCTTGGAGTTAACCTCAAGATGATTACATCTAAATACCCCACAATTAAGGGCACTAATTT

    L  G  V  N  L  K  M  I  T  S  K  Y  P  T  I  K  G  T  N  F  232

781 TGATTTGCCACATGTTGTTCAACATGCCCCTTCCTATCCTGGGGTGGAACATGTTGGGGG

    D  L  P  H  V  V  Q  H  A  P  S  Y  P  G  V  E  H  V  G  G  252

841 AGATATGTTTGAAAGTGTTCCAGAAGGAGATGCTATTTTTATGAAGTGGATTCTTCATGA

    D  M  P  F  S  V  P  E  G  D  A  I  F  M  K  W  I  L  H  D  272
```

FIG. 4 (4/5)

```
901  CTGGAGTGATAGTCACAACCTCAAGTTGCTAAAGAACTGCTACAAGGCTCTACCAGACAA

       W  S  D  S  H  N  L  K  L  L  K  N  C  Y  K  A  L  P  D  N  292

961  TGGAAAGGTGATTGTTGTTGAGGCCATTTTACCAGTGAAACCAGACATTGACACCGCAGT

       G  K  V  I  V  V  E  A  I  L  P  V  K  P  D  I  D  T  A  V  312

1021 GGTTGGCGTTTCGCAATGTGATTTGATCATGATGGCTCAAAATCCTGGAGGCAAAGAGCG

       V  G  V  S  Q  C  D  L  I  M  M  A  Q  N  P  G  G  K  E  R  332

1080 ATCGGAAGAGGAGTTTCGAGCCTTGGCTACTGAAGCTGGATTCAAAGGCGTTAACTTAAT

       S  E  E  E  F  R  A  L  A  T  E  A  G  F  K  G  V  N  L  I  352

1141 ATGTTGTGTCTGTAATTTTTGGGTCATGGAATTCTGCAAGTAGATTTCTACTGTACATTG
```

C C V C F P W V M E F C K *

1201 AGTTTCTACTACTCTTGAGTATCCATTTATGGCAATCTGGGACTGGAATTGCAGCTTAGT

1261 CCAGATTGAACATTGATATTCCTAATAATATTTCTATTATTTCCCTTGTTTATTTCTCTT

1321 GTATGAAAGGATGTCATTTTGAGTATTGATAATCATGTTCTCTAGGACAGAAATTGTAAC

1381 TTTGTCCAACTTTATTGATATTCCTAGTAAGATTTATATGACATGTGTCTCTGGTTTGAG

1441 AAGAGTTTCAATATCTAAAAAAAAAAAAAAA

FIG. 4 (5/5)

FIG. 5

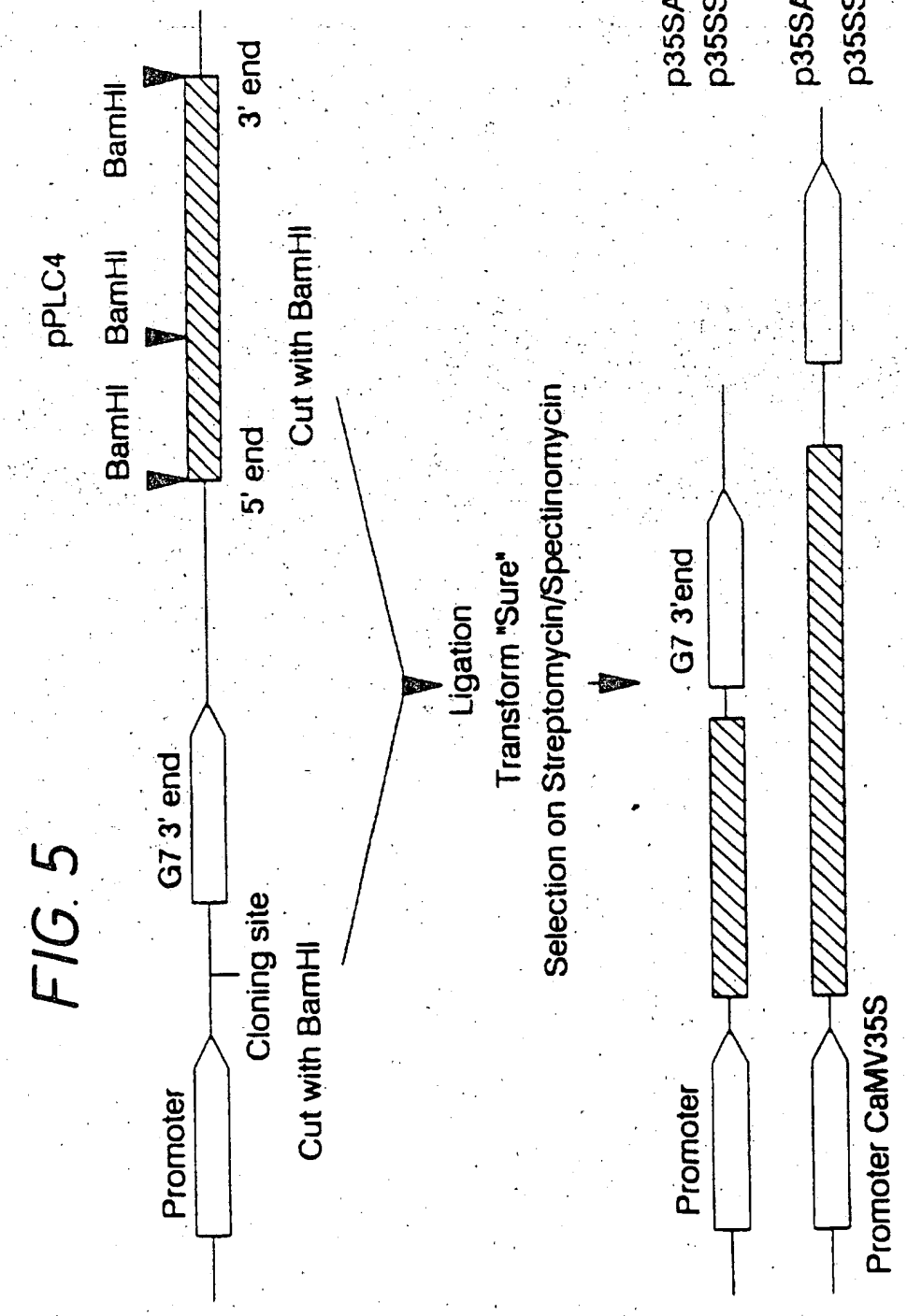
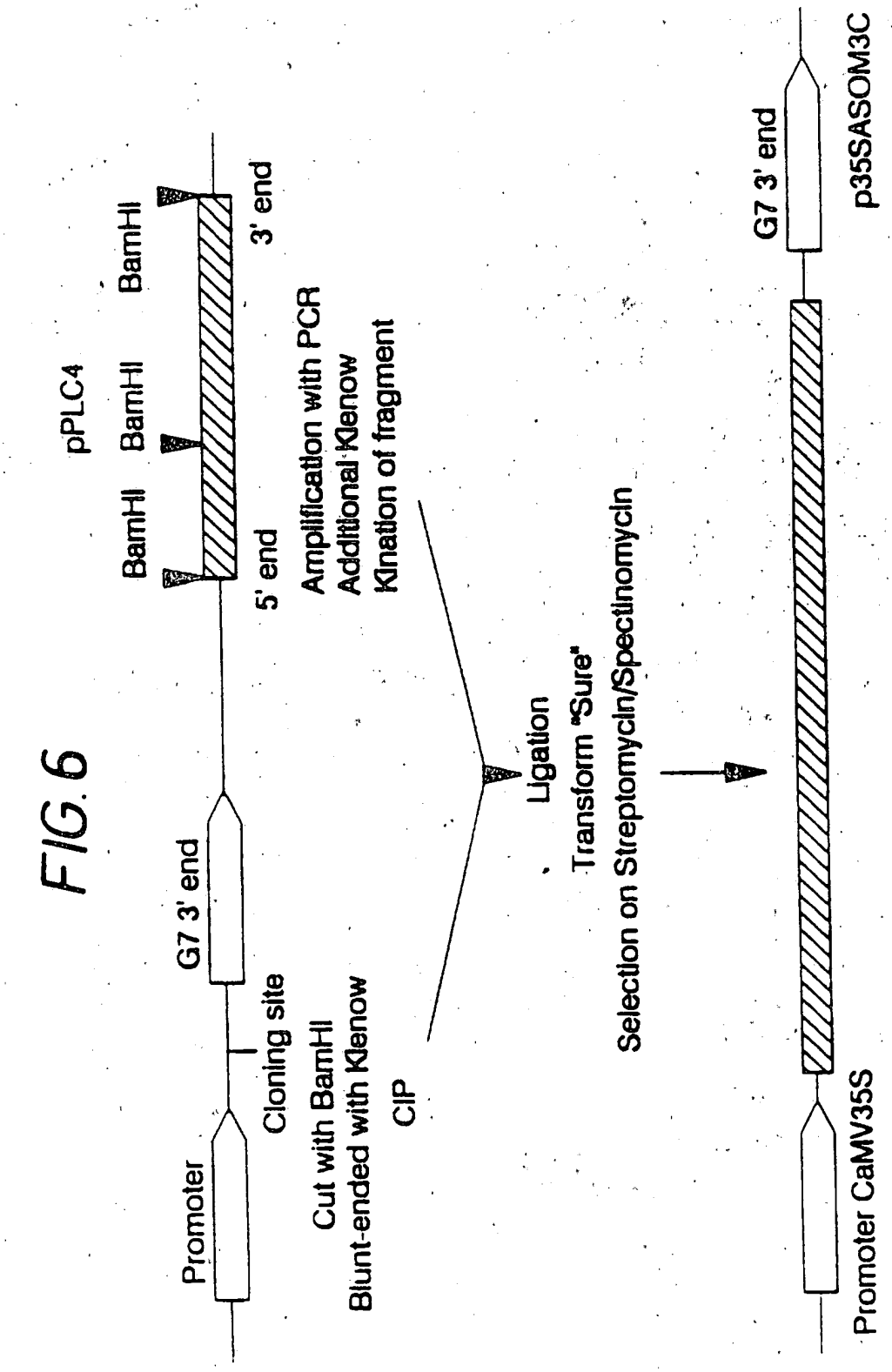

pPLC4
BamHI
BamHI
BamHI
3' end
5' end
Cut with BamHI
G7 3' end
Cloning site
Promoter
Cut with BamHI
Ligation
Transform "Sure"
Selection on Streptomycin/Spectinomycin
p35SASOM3A
p35SSOM3A
p35SASOM3B
p35SSOM3B
G7 3'end
Promoter
Promoter CaMV35S

FIG. 6


pPLC4
BamHI
BamHI
BamHI
3' end
5' end
Amplification with PCR
Additional Klenow
Klnation of fragment
G7 3' end
Cloning site
Promoter
Cut with BamHI
Blunt-ended with Klenow
CIP
Ligation
Transform "Sure"
Selection on Streptomycin/Spectinomycin
G7 3' end
p35SASOM3C
Promoter CaMV35S

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01640

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/54; C12N15/82; C12N15/11; C12N5/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	PLANT PHYSIOLOGY. vol. 97, no. 1, September 1991, ROCKVILLE, MD, USA. pages 7 - 14 GOWRI, G., ET AL. 'Stress responses in Alfalfa (Medicago sativa L.)' see the whole document particularly page 13 right col. last para	1
Y		2-11
X	J. CELL. BIOCHEM. SUPPL.,MEETING HELD JAN 10-17, 1991. vol. 15A, 1991, page 144 CAMPBELL, W.H., ET AL. 'Seasonal expression of a lignin specific O-methyltransferase cloned from aspen developing secondary xylem' see the whole document	1

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 DECEMBER 1992	[illegible]

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PLANT PHYSIOLOGY, (SUPPL.) vol. 93, no. 1, May 1990, ROCKVILLE, MD, USA. page 15 BUGOS, R.C., ET AL. 'Cloning and expression of lignin bispecific O-methyltransferase' see abstract 83	1
Y	CA,A,2 005 597 (SCHUCH) 15 June 1990 see page 13, line 14 - line 34	2-5,10, 11
Y	BIOLOGICAL ABSTRACTS vol. 73 , 1982, Philadelphia, PA, US; abstract no. 64323, MARIGO, G., ET AL. 'Biosynthesis of carbon-14-labeled ferulic acid and sinapic acid' see abstract & J. LABELLED COMPD. RADIOPHARM. vol. 18, no. 5, 1981, pages 695 - 702	6
Y	BIOLOGICAL ABSTRACTS vol. 84 , 1987, Philadelphia, PA, US; abstract no. 19249, HERMANN, C., ET AL. 'Enzymatic synthesis of lignin: Purification to homogeneity of the three O-methyltransferases of tobacco and production of specific antibodies' see abstract & ARCH. BIOCHEM. BIOPHYS. vol. 253, no. 2, 1987, pages 367 - 376	7-9
P,X	PLANT PHYSIOLOGY. vol. 98, no. 2, February 1992, ROCKVILLE, MD, USA. pages 796 - 797 DUMAS, B., ET AL. 'Nucleotide sequence of a complementary DNA encoding O-methyltransferase from poplar' see the whole document	6

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	BIOLOGICAL ABSTRACTS vol. 94 , 1992, Philadelphia, PA, US; abstract no. 89217, JAECK, E., ET AL. 'Regulation of enzymes involved in lignin biosynthesis: Induction of O-methyltransferase mRNAs during the hypersensitive reaction of tobacco to tobacco mosaic virus' see abstract & MOL PLANT-MICROBE INTERACT. vol. 5, no. 4, 1992, pages 294 - 300	7,8

O,P, X	J. CELL. BIOCHEM. SUPPL., MEETING HELD APRIL 10-16, 1992. vol. 16F, 1992, page 219 NI, W., ET AL. 'Modification of lignin biosynthesis by genetic manipulation of caffeic acid O-methyltransferase' see abstract Y219	3-5,10, 11

O,P, X	PLANT PHYSIOLOGY, (SUPPL.) vol. 99, no. 1, May 1992, ROCKVILLE, MD, USA. page 19 PODILA, G.K., ET AL. 'Antisense expression of an aspen O-methyltransferase construct in transgenic tobacco via Agrobacterium' see abstract 110	2,3,5, 10,11

A	PLANT PHYSIOLOGY. vol. 96, no. 2, June 1991, ROCKVILLE, MD, USA. pages 577 - 583 LAGRIMINI, L.M., ET AL. 'Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase' see the whole document	4,10,11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201640 SA 64222

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 08/12/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA-A-2005597	15-06-90	None	